

**EVALUATION OF IMMUNOMODULATORY ACTIVITY OF ETHANOLIC  
EXTRACT OF *ANNONA RECTICULATA* FRUITS**

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**Submitted by:**

**FATHIMA SHAMEELA P K**

**Reg. No:261425721**

**Under The Guidance Of**

**Mr.A.SURESH.M.Pharm.,(Ph.D).**



**DEPARTMENT OF PHARMACOLOGY**

**JKKMMRF'S – ANNAI JKK SAMPOORANI AMMAL  
COLLEGE OF PHARMACY  
B. KOMARAPALAYAM–638 183.  
TAMIL NADU**

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## 1. INTRODUCTION

Human beings are living in an open environment which is heavily contaminated with many pollutants. They are constantly exposed to varieties of physical, chemical, and environmental factors that cause adverse effects and damages on human. In nature our body has different strategy to control the above mentioned factors, and man is constantly trying to find and identify remedies for these problems. Plants are used since very ancient time to control different types of problem associated with human. There are reports that plants contain remedies for various diseases including AIDS, cancer, and diabetes (**Ongradi *et al.*, 1999, Vijayvargia *et al.*, 2000, Wu *et al.*, 2001**). The use of plant-based medicines for healing is an ancient and universal as medicine itself. Until the dawn of this century, natural products have served as the mainstay of all medicines world-wide. Although herbalism has declined in the West, it continues to exist throughout the developing world. According to WHO, over 70% of the world population still relies on herbal remedies for their health care needs (**Atta-ur-Rahman and Choudhary, 1999**). Today there are a huge number of chemical substances derived from plants that are considered as important drugs currently in use in one or more countries in the world

Many of the plant products exert some effect on immune system. They either enhance immune response to help body clear undesirable agents from body or suppress immune response to control deterioration in the body. Compounds that alter immune response are considered Immuno modulatory agent.

**The ubiquitous enemy:**

Microbes are able to survive on animal and plant products by releasing digestive enzymes directly absorbing the food, and / or by growth on living tissues (Extra cellular), in which case they are simply bathed in nutrients. Other microbes infect (Invade) animal / human cells (intracellular), where they not only survive, but also replicate, utilizing host-cell energy sources.

Both extra cellular and intercellular microbes can grow, reproduce and infect other individuals. They are many different species of microbes and larger organisms (such worms) which invade humans, some of which are relatively harmless and some even helpful (e.g. E.coli in our intestines). Many other causes diseases (human pathogens, and there is a constant battle invading microbes and immune- system. Some microbes can even cause the death of their hosts, although this should not be the property of the most successful microbes. The range of organisms that can infect humans. E.g. HIV.

**Range of infectious organisms**

Worms (Helminthes)	: e.g. Tape worms, Filaria
Protozoan	: e.g. Trpanosomes, Leishmania, Malaria
Fungi	: e.g. Candida, Aspergillus.
Bacteria	: e.g. E.coli, Staphylococcus, Streptococcus, Mycobacteria
Viruses	: e.g. Polio. Pox viruses, Influenza, Hepatitis-B, HIV

**External defenses****I) Physical barriers to entry of microbes**

Before a microbe or parasites can invade the host and causes infections, it must first attach to and penetrate the surface epithelial layers of the body. Organisms gain entrance into the body by an active or passive means. For example, they might burrow through the skin, or be ingested in food, inhaled into the respiratory tract or penetrate through an open wound. In practice, most microbes take advantage of the fact that we have to breathe and eat to live and therefore enter the body through the respiratory and gastrointestinal tract. Whatever their point of entry, they have to pass across physical barriers such as the dead layer of the skin or living epithelial cells layers which lines the cavities in contact with the exterior such as the respiratory, genitourinary tract or gastrointestinal tract . In fact, the main entry of microbes into the body is via these tracts.

**ii) Secretions**

Verities of secretions at epithelial surfaces are important in defense. The overall aim is to provide a hostile environment for microbial habitation. Some substances are known to directly kill microbes e.g. lysozymes by digesting protelglycans in bacterial cell walls: other competes for nutrients (e.g. transferring, Fe), and others interfere with ion transport (e.g. NaCl). Mucus (containing mucin) secreted by the mucosal epithelial cells coat their surfaces and make it difficult for microbes to contact and bind to them-a prerequisite for entry in to the body.

The washing action of tears, Saliva and urine also help to prevent attachment of microbes to the epithelial surfaces. In addition, tears and saliva contain IgA antibodies which are secreted across epithelial cells and prevent the attachment of microbes. These antibodies are also present in genitourinary tract. Gastrointestinal,

respiratory epithelia and Phagocytes throughout the body are also known to produce a number of small peptides which have potent anti-bacterial properties (Peptide antibiotics).

### **iii) Microbial products and competition**

Normal commensals (non pathogenic bacteria) also help to protect from infection. These non pathogenic microorganisms are found on the skin, in the mouth and in the reproductive and gastrointestinal tract. The gastrointestinal tract contains many billions of bacteria that have a symbiotic relationship with the host. These bacteria help to prevent pathogens from colonizing and releasing antibacterial substances such as colicins (anti bacterial proteins) and short- chain fatty acid.

## **1.1 The Immune System**

Immune system is a remarkably sophisticated adaptive defense system within vertebrates, to protect them from invading agents and cancer. It is able to generate varieties of cells and molecules capable of recognizing and eliminating limitless varieties of foreign invaders. Functionally the immune system is divided into two interrelated activities, recognition and response. Immune system is able to recognize foreign substances, and discriminate foreign molecules from own cells and proteins. Once foreign molecule or organism is recognized, immune system responds to eliminate or neutralize these by utilizing the initial recognition information. The same response could be repeated if the immune system encounters the same antigen (memory response) (**Kuby, 1994**). On the other hand the immune system at certain times attack or mistakenly respond to the own tissues, that may result in autoimmune diseases such as multiple sclerosis, rheumatoid arthritis and juvenile

diabetes (Masakuni and Toru, 1994). Similarly an immune system responds to a transplanted organ from a donor and result in Graft vs. host disease (Kuby, 1994).

Immune system is a complex organization of white blood cells, antibodies, and blood factors that protects the body from foreign invaders, while simultaneously maintaining self-tolerance. A series of specialized epithelial and stromal cells also provide the anatomic environment which regulate various functions of immune system by secreting several critical factors. The immune system is a network of cells, tissues, and organs that work together to defend the body against attacks by “foreign” invaders. These are primarily microbes (germs) -tiny, infection-causing organisms such as bacteria, viruses, parasites, and fungi. Because the human body provides an ideal environment for many microbes, they try to break in. It is the immune system’s job to keep them out or, failing that, to seek out and destroy them.

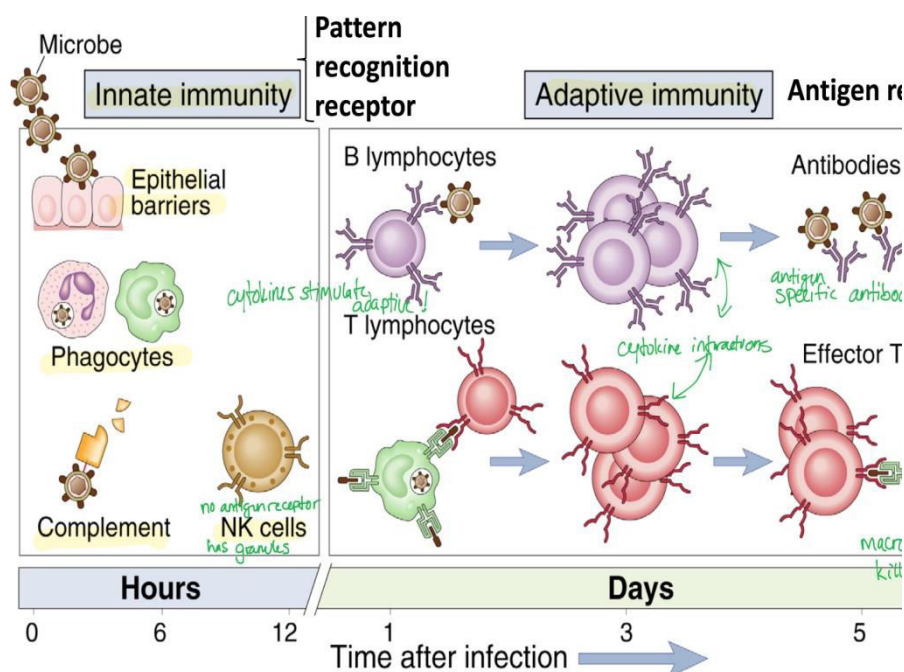


Fig. 1 Special Cells Responsible For Immune System

The immune system is a system of biological structures and processes within an organism that protects against disease. Disorders of the immune system can result in autoimmune diseases, inflammatory diseases, cancer and immunodeficiency. Immuno modulation is a procedure which can alter the immune system of an organism by interfering with its functions; if it results in an enhancement of immune reaction, it is named as an immunostimulative drug which primarily implies stimulation of non-specific system. Immunosuppressant implies mainly to reduce resistance against infections, stress and may occur on account of environmental or chemotherapeutic factors. Immunostimulation and immunosuppression both need to be considered in order to regulate the normal immunological functioning. Hence both immune stimulating agents and immune suppressing agents have their own standing, so search for better agents exerting these activities is becoming the field of major interest all over the world.

A number of Indian medicinal plants and various ‘Rasayana’ have been claimed to possess Immunomodulatory activity .The use of plant products as immunomodulators is still in a developing stage. A variety of plant-derived materials such as polysaccharides, lectins, peptides flavonoids and tannins have been reported to modulate the immune system. Since ancient times, several diseases have been treated by administration of plant extracts based on traditional medicine. Natural adjuvants, synthetic agents, antibody reagents are used as immunosuppressive and immunostimulative agents. But there are major limitation to the general use of these agents such as increased risk of infection and generalized effect throughout the immune system.



## **1.2 The Organs of the Immune System:**

The immunological system is comprised of the lymphoid tissues and organs of the body. Immune cells have two main sites for their origin, and proliferation or activation (where they are located to target the foreign particles). These are the primary lymphoid organ and the secondary lymphoid organ (**Levinson and Jawetz, 1992**).

### **a) Primary lymphoid Organ**

Thymus gland and the bone marrows produce the specialized lymphocytes (T-cells and B-cells) and dispatch them through the lymph vessels to the secondary organs for their maturation, proliferation and storage. The bone marrow (soft tissue located in the cavities of the bones), produce and differentiate blood cells. It is the source of stem cells, which differentiate leukocytes. From the bone marrow lymphocytes (T Cell ) are sent to the thymus gland to mature, and are then stored in the secondary organs of the lymph system and in the blood stream. B-cells get matured and differentiated in the bone marrow then send to the secondary organs (**Reeves, 1987**).

### **b) Secondary Organs**

Secondary Organs are the lymph nodes, spleen, tonsils, and Peyer's patches in the small intestines, liver, and appendix. They are the locations where the molecular parts of the immune system gather in readiness to do battle with germs, viruses, and allergens (**Reeves, 1987**).

## **Specialized Cells of the immune system**

There are several types of cells responsible for specialized function in the immune system, they are mainly classified as two major cell type: leucocytes and lymphocytes.

### **Leucocytes**

Leucocytes (White blood cells) are the main cells of the immune system provide either innate or specific adaptive immunity. They are motile with specialized functions. The number of leucocytes in normal blood ranges between 4,500 and 11,000 per mm<sup>3</sup>. Most of the leucocytes are outside the circulation, and the few in the bloodstream are in transit from one site to another. They are further classified into granulocytes, monocytes, and lymphocytes (**Mosman and Coffman, 1989**).

The most numerous of the leucocytes are important mediators of the inflammatory response. There are three types of granulocytes: neutrophils (50-70 %), eosinophils (1-4 %), and basophils (0.5 %) (**Kuby, 1994**).

### **Neutrophils**

One of the major types of cells that recruit to ingest, kill and digest pathogens. They live only a few hours in the blood, some migrate to the tissues to areas of infection or injury. Neutrophils engulf and digest bacteria and other microorganisms and microscopic particles.

**Eosinophils**

Eosinophils are also motile and phagocytic, and migrate into the tissues. They are particularly important in the defence against parasites, and they participate in hypersensitivity and inflammatory (allergic) reactions. Their cytotoxicity is mediated by cytoplasmic granules.

**Basophils**

They have many large cytoplasmic granules, which contain heparin and histamine. When aggravated, they release histamine and other mediators that involve in allergic reactions. They also produce cytokines. Basophils display high affinity surface membrane receptors for Ig E antibodies (**Roitt *et al.*, 1998**).

**Monocytes**

Monocytes are also produced in the bone marrow. They constitute up to 10 % of the blood leucocytes. However, the majorities leave the blood after a few hours and migrate into almost all tissues, where they develop into macrophages. Both monocytes and macrophages are highly adherent, motile and phagocytic. They marshal and regulate other cells of the immune system. They serve as antigen processing-presenting cells and act as Cytotoxic cells when armed with specific IgG antibodies.

**Natural killer (NK) cells**

The term natural is used because NK cells are present without previous immunization and act immediately on target cells. NK cell lacks antigen specific receptors that are typical of B and T cells. They are part of innate immunity,

important in elimination of tumours and virus-infected cells. NK cells are derived from the bone marrow and can be distinguished by characteristic of azurophilic granules in their cytoplasm. NK cells destroy the target not only by the NK cell-mediated cytotoxicity but can also mediate antibody-dependent cellular cytotoxicity (ADCC) via binding to the Fc portion of IgG on target cells. They represent only a small fraction of peripheral blood cells and a small fraction of lymphoid cells in the spleen and other secondary lymphoid tissues. NK cells have no antigen-specific receptors. Their Cytotoxic activity is inhibited by encounter with self-MHC molecules through inhibitory receptors on their surface that recognize class I. They thus kill self cells that have down regulated class I molecule expression (Cederbrant *et al.*, 2003). Treatment of target cells with IFN- $\gamma$  increases expression of MHC class I, whereas this increased expression of MHC class I increases the susceptibility to TCTL-cell-mediated lysis, it actually protects against NK-mediated lysis. Thus there are generally opposite requirements of TCTL and NK cells for targeting cells, TCTL cells require MHC class I recognition for killing and NK cells are active against targets that do not express MHC class I. In addition to the anti-tumor activities of NK cells, they have been reported to play an important role in limiting the growth or spread of a variety of microbial infections. NK cells may also be involved in regulating haematopoiesis as well as immunoregulatory role via the production of a variety of cytokines (Sell, 2001).

### **Lymphocytes**

Lymphocytes are small white cells found in lymphoid organs and in the blood. They get to the blood stream from the lymph nodes, which function to trap antigens and filter them out of the lymph fluid. Lymphocytes constitute about 25-50 % of the

blood leucocytes. They are non-motile and enter the circulation through lymphatic channels. Some lymphocytes leave and reenter the circulation, surviving for many years. They are found in large numbers in the secondary lymphoid organs. When stimulated by antigen for example or a microbe, lymphocyte divides several times into daughter cells and eventually generates a clone of identical lymphocytes. Some of these cells remain in the circulation; others patrol the tissues of the body. The larger number of lymphocytes capable of reacting to the same microbe is responsible for the immunological memory that is manifested, if the body encounters the same microbe later in life. Lymphocytes are divided into two main classes according to their origin and differentiation. T-Lymphocytes which are produced in the bone marrow pass through the thymus to get mature. The other class is the B-lymphocytes; they do not pass through thymus but get mature in gut associated lymphoid tissues (GALT), which in mammalian are equivalent to the bursa of Fabricius in birds (**Mosman and Coffman, 1989**).

- I. **T – Lymphocytes:** There are two major classes of T-lymphocytes: T helper (Th) and T- cytotoxic lymphocytes (Tc).

**T helper (Th) lymphocytes** regulate the antibody-forming function of B-lymphocytes and participate in rejection of transplants. They possess CD4 surface molecules (also called CD4 cells). T helper cells are functionally further subdivided into at least two types, Th-1 and Th-2.

The other T lymphocytes those involved in the defenses against virus infection, is called T-cytotoxic lymphocytes (Tc) or T-suppressor (Ts).

**Cytotoxic T (Tc)** cells are capable of destroying a target cell, that is infected with virus or that expresses some form of foreign antigen. These cells are the major immune effector of the cellular immune response. They also express CD8 molecules (Levine *et al.*, 1998).

**T-suppressor (Ts)** cells act to diminish helper T-cell activity; they directly kill virus infected or cancer cells when the battle is over. They express CD8 molecules. In contrast to helper T cells, Ts cells down-modulate immune responses. Thus, the combination of helper and suppressor cells determines the level of the immune response to any specific antigen (Levine *et al.*, 1998).

## **II. B- lymphocytes**

They have a relatively short life span compared to T-cells. As B-cells mature, they turn into antibody-producing plasma cells found in lymph nodes and in the spleen. Once the B-cells have created a specific antibody to attack a specific pathogen, their primitive intelligence remembers this information and will know it later (Levine *et al.*, 1998)

### **The ubiquitous enemy**

Microbes are able to survive on animal and plant products by releasing digestive enzymes directly absorbing the food, and / or by growth on living tissues (Extra cellular), in which case they are simply bathed in nutrients. Other microbes infect (Invade) animal / human cells (intracellular), where they not only survive, but also replicate, utilizing host-cell energy sources.

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(such worms) which invade humans, some of which are relatively harmless and some even helpful (e.g. E.coli in our intestines). Many other causes diseases (human pathogens, and there is a constant battle invading microbes and immune- system. Some microbes can even cause the death of their hosts, although this should not be the property of the most successful microbes. The range of organisms that can infect humans e.g. HIV.

### **Immunity**

The system consists of innate components that act rapidly (within hours) but non-specifically (Innate Immunity - involves granulocytes, macrophages and natural killer (NK) cells for examples) and adaptive or acquired components that act specifically, but need time to respond (adaptive immunity - mediated by lymphocytes, initiate in 4-7 days).

### **Innate immunity**

Innate immunity is considered to be antigen independent that occurs without prior exposure to antigens. It can be triggered upon the initial encounter with a foreign substance; its components are often called the first line of defence. Skin is considered as a component of innate immunity. Similarly, the following bodily functions contribute to host defence and are considered parts of innate immunity: the lysosomal enzymes in salivary, lacrimal and vaginal secretions, which have bacteriostatic properties, the cough reflex, which is an important mechanism to clear the bronchial passages of irritants and potential infectious microbes, and the fever response, which is an important reaction to an infection. Important components of the innate immune defence system include phagocytic cells such as Neutrophils, macrophages, NK cells, and the soluble products, type I interferon and complement.

## **Receptors of the Innate Immune Response**

In order to detect PAMPs or DAMPs, cells need tools to recognize them. These tools are protein receptors that can be found on the cell surface as well as internally. In general, they are called pattern recognition receptors or PRRs. These receptors come in families consisting of multiple members. Receptors that recognize PAMPs include the Toll-like receptors (TLRs), the C-type lectin receptors (CLRs), the NOD-like receptors (NLRs), RIG-I-like receptors (RLRs) and invariant T cell receptors. DAMP receptors are not so clear-cut. TLRs have been implicated as well as the receptor for advanced glycation end products (RAGE). Also the purinergic receptors that recognize ATP would also fall into this category

### **Toll-like Receptors**

These receptors are found on most cells of the body. They recognize a variety patterns associated with a number of pathogens including virus-associated nucleic acids; bacterial-associated cell wall components, protein, ribosomal RNA and DNA; and protozoan-associated proteins. The majority is found extracellular, but a number are also found intracellular. When stimulated they activate the transcription factor NF $\kappa$ B, which is essential for activating a cell's immune functions and set off a signal cascade via MAP kinase (a phosphorylating enzyme).

### **C-type Lectin Receptors**

These receptors are specialized in recognizing carbohydrate structures, such as the sugar mannose, which is a common component of fungal cell walls. Thus, these receptors are found on the cell surface. Though much of the literature involves their expression on immune cells, reports of CLR variants on non-immune cells can also be found. On the phagocytic cells, it is known that they can participate in endocytosis, the engulfment of particles or pathogens and respiratory burst.<sup>15</sup> Some



also appear to initiate signal cascades similar to TLRs leading to NF $\kappa$ B and MAP kinase activation, but it also appears that they can work in concert with TLRs, enhancing or inhibiting their function.

### **NOD-like Receptors**

These receptors are found in the cytoplasm of cells. Traces of their expression is found in most organs of the body and it is probably safe to say that most immune cells express at least some members of the NLR family. These receptors are designed to detect intracellular bacteria and, possibly, endogenous stress molecules and allow the cell to produce one of the most potent inflammatory mediators, Interleukin (IL)-1 $\beta$ .<sup>17</sup>

### **RIG-I-like Receptors**

Like NLRs, RLRs are also found in the cytoplasm of a cell. Instead of detecting bacterial products, these receptors help detect viral infection. <sup>18</sup>They do this by binding to RNA produced during viral replication. Working together with nucleic-acid detecting TLRs, they lead to NF $\kappa$ B, MAP kinase activation and activation of Interferon regulatory factor (IRF) transcription factors.<sup>19</sup>The IRF transcription factors are necessary to produce cytokines specialized for the control of viral infections. Cytokines are small, secreted proteins used as messengers between cells, which alert surrounding immune cells about danger.

### **Immune Cells of the Innate Response**

Under epithelial layers are resident macrophages, neutrophils, dendritic cells, NK cells, mast cells and a number of T cell-related cells.<sup>20</sup>

### **Macrophages**

The name macrophage is derived from Greek, meaning - large eaters|. Their main function is to phagocytize (engulf) pathogens and particles. It does this by

wrapping its plasma membrane around particles until they are enveloped and pinched off to form an endosome inside the cell. Once inside the cell, the endosome merges with a lysosome that contains enzymes and acids that can digest the contents. Macrophages also have the ability to generate a respiratory burst, which is a release of oxygen radicals that damage surrounding pathogens and cells. They also can alert and attract other immune cells through inflammatory cytokine release.

### **Neutrophils**

Neutrophils are the main foot soldiers of the innate immune response and are certainly the most abundant. They also have a wide arsenal of tools to deal with invaders. Like macrophages, neutrophils can phagocytize particles, release a respiratory burst and produce inflammatory cytokines. Unlike macrophages, neutrophils have the internal caches of anti-microbial substances called granules.<sup>22</sup>

### **Dendritic Cells**

Dendritic cells are also phagocytic cells, but they have the special ability of initiating an adaptive immune response (will be discussed later). Unlike neutrophils and macrophages, Dendritic cells or DCs are not simple foot soldiers. Instead, they function more as spies and provide intelligence about invaders to T cells through a phenomenon called —antigen presentation and through cytokine production.

### **NK Cells**

The NK stands for Natural Killer and the name implies their function. These cells, however, do not kill pathogens directly. Instead, these cells have the ability to recognize when other cells are harboring internal pathogens using special receptors and then kill them. Situations where this might occur is during viral and mycobacterial infections. These pathogens easily reside in host cells, finding ways to block lysosome fusion and their own destruction.

**Mast Cells**

Mast cells are the cells that are responsible for the classic signs of inflammation, which include redness, swelling and heat. Though well known for their association with allergy, they also can detect PAMPs and DAMPs through receptors and become immunologically active. Mast cells exert their functions mainly through cytokine and granule release. Unlike neutrophils, which release antimicrobial substances, mast cells release histamine and heparin. Histamine is well known for its vasodilator function and ability to allow fluid to leak between cells, causing redness and swelling. It also causes inflammatory itching by triggering neurons (unmyelinated C-fibers) responsible for the itch feeling. Heparin prevents blood coagulation.

**T cell-like Cells**

Most T cells are part of the adaptive immune response as they have adaptive T cell receptors (receptors that learn to recognize pathogens). NK T cells and  $\gamma\delta$  T cells, however, use invariant T cell receptors (receptors that do not rearrange) or semi-invariant T cell receptors and participate in the innate immune response.

NK T cells are similar to the NK cells mentioned above. Not so much in function, but more in how they look. These cells share many of the same surface protein markers. NK T cells, however, do not kill compromised cells. Instead, they are quick cytokine producers. In doing so, they quickly notify all surrounding cells that there is problem when they recognize PAMPs presented to them via dendritic cells.

The  $\gamma\delta$  T cells are important for innate immune reactions and the adaptive immune response as they have invariant and variant T cell receptors. Their precise

function remains unclear, but they can secrete cytokines and, like the NK T cells above, participate in alerting and strengthening local immune responses.

### **Non-cellular Systems of the Innate Immune Response**

Besides cells, there are also defenses in your body that are ready to react to pathogens as soon as they are encountered, much like booby traps. These systems rely on small proteins that are found within the bodily fluids.

#### **Complement System**

The liver synthesizes the proteins of the complement system and they work in concert to aid in phagocytosis, bacteria lysing and immune cell attraction. One can visualize it as a self-assembling machine that starts to assemble as soon as the first proteins are bound and in place. The complement\_ machine is known to be initiated by three different pathways: the classical pathway, the alternative pathway and the lectin pathway. The classical pathway is triggered when antibodies are bound to a pathogen. The alternative pathway is triggered when the victim is unable to block the cascade (normal cells can, while pathogens cannot). The lectin pathway uses free lectin proteins (lectins are proteins that bind sugars) to bind sugars associated with bacterial cell walls).

#### **Acute Phase Proteins**

These proteins are also produced by the liver and especially during inflammation when pro-inflammatory cytokines are produced. Many are designed to coat pathogens and have chemotactic properties (have the ability to attract cells). Some inhibit microbial growth by sequestering iron from the environment. The lectins from the lectin pathway of complement activation are considered acute phase proteins.

## Anti-microbial Peptides

Often called —defensins, these peptides function as natural antibiotics and are produced by cells that guard the external surfaces and internal surfaces such as the skin and the gastrointestinal system. In the skin, the main sources are keratinocytes, mast cells, neutrophils, sebocytes and eccrine epithelial cells. In the intestines, one of the main producers are the Paneth cells of intestinal crypts.

## Acquired immunity

Acquired immunity is antigen dependent and comprises of all the specific immunological reactions associated with lymphocytes. Acquired immunity is subdivided into two effector arms, humoral immunity and cell mediated immunity. Humoral immunity is mediated by soluble protein molecules known as antibodies that are produced by B lymphocytes. Antibodies are specifically recognized microbial antigens, neutralize the infectivity of the microbes and target microbes for elimination by various effector mechanisms.

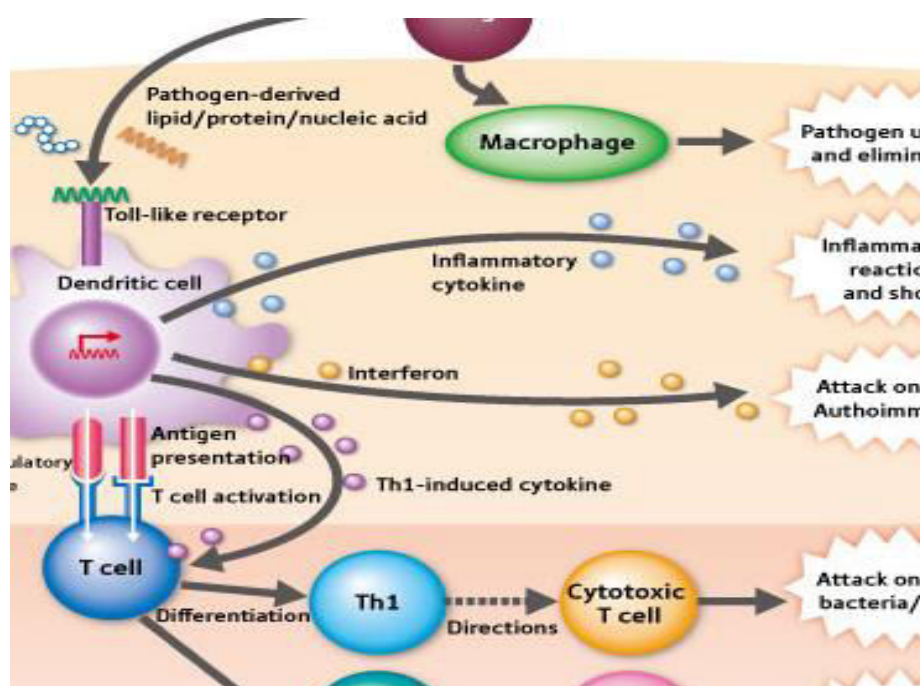


Fig.2 Mechanism of Immunity.

**Humoral immunity**

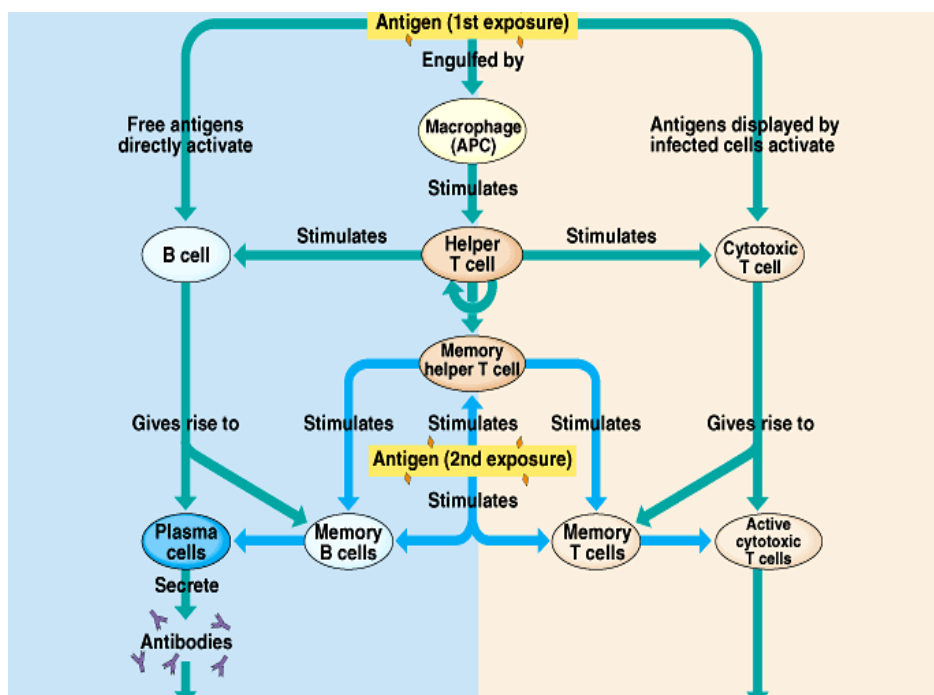
The defence mechanism against extracellular microbes and their toxins, because secreted antibodies can bind to these microbes and toxins and assist in their elimination. Antibodies themselves are specialized and different types of antibodies may activate different effector mechanisms.

**Cell mediated immunity**

It is the result of the activity of many leukocyte actions, reactions, interactions that range from simple to complex. This type of immunity is dependent on the actions of the T (Thymus) lymphocytes, which are responsible for a delayed type of immune response. The T lymphocyte becomes sensitized by its first contact with a specific antigen. It is also called cellular immunity.

**1.4 Innate immune system**

**Innate immune system** The term “innate” refers to that part of the immune system with which we are born; that is, it does not change or adapt to specific pathogens(unlike the adaptive immune system). The innate immune system provides a rapid first line of defence, to keep early infection in check, giving the adaptive immune system time to build up a more specific response. Innate immunity consists primarily of a chemical response system called complement, and the endocytic and phagocytic systems, which involve roaming “scavenger” cells, such as macrophages, that detect and engulf extracellular molecules and materials, clearing the system of both debris and pathogens.



**Fig.3 Immune Response Structure**

### 1.5 Adaptive immune system

The adaptive immune system is so-called because it adapts or “learns” to recognize specific kinds of pathogens, and retains a “memory” of them for speeding up future responses. The learning occurs during a primary response to a kind of pathogen not encountered before by the immune system. The primary response is slow, often first only becoming apparent several days after the initial infection, and taking up to three weeks to clear an infection. After the primary response clears an infection, the immune system retains a memory of the kind of pathogen that caused the infection. Should the body be infected again by the same kind of pathogen, the immune system does not have to re-learn to recognize the pathogens, because it “remembers” their specific appearance, and will mount much more rapid and efficient secondary response. The secondary response is often quick enough so that there are no clinical indications of a re-infection. Immune memory can confer

protection up to the life-time of the organism (measles is a good example in this regard). The adaptive immune system primarily consists of certain types of white blood cells, called lymphocytes, which circulate around the body via the blood and lymph systems (**Hofmeyr Steven, 2000**).

### **T helper Cells and Their Education**

T helper cells or Th cells are crucial cells in the adaptive immune response and they are characterized by a surface protein called, CD4. They hold the key to initiating the functions of cytotoxic T cells<sup>33</sup> and B cells<sup>34</sup>. Furthermore, they can also increase the efficacy of macrophages

Th cells interact with the MHC class II/peptide complexes presented by antigen presenting cells through its receptor, called the T cell receptor (TCR). If a T cell has never before seen antigen, it is called a naïve T cell. In this situation, the T cell will need instruction from a professional antigen presenting cells, usually a DC, about how to perform its function. DCs do this through cell surface proteins call co-stimulatory molecules and through cytokine expression. This process is consists of three main signals. The first signal is the antigen recognition; the second signal is co-stimulation and the third cytokine exposure. This whole process is referred to as \_ priming of the naïve T cell. Once primed, the T cells begin to divide; a process that is referred to as expansion or proliferation.

The most important set of co-stimulatory molecules is CD80 or CD86 on the DC and CD28 on the T cells. This second signal is necessary to tell the Th cell that there is a problem. If signal one is given without this second signal, the T cell will assume that the antigen is actually harmless and become non-responsive in a process called —anergy<sup>36</sup>. Only a DC that has encountered a PAMP or another danger



signal will express CD80 or CD86 on its surface reassuring the Th cell that there is, indeed, a problem.

Signal three is the secretion of cytokines of the DC. There are several cytokines important for Th cell education. The most important ones are IL-4, IL-12, IL-6, TGF $\beta$  and IL-10. Th cells will differentiate into different types of Th cells depending on which cytokines prevail. The main types of Th cells are T helper 1 (Th1) cells, T helper 2 (Th2) cells, T helper 17 (Th17) cells, and induced regulatory T cells (iTreg).

### **Th Cell Subtypes**

Each Th cell subtype has its own unique set of skills. One could almost see differentiation as an occupation. Just like an athlete will choose to develop her body and a scientist will choose to develop her mind. In humans, these choices are reflected at the level of gene transcription and protein expression. The athlete will stimulate muscle growth and the scientist develops the cerebral cortex of the brain. It's the same for Th cell differentiation. The four main subtypes of Th cells are listed. There are, however, rare forms that have been observed that are not listed and Th cells, much like humans, can fall into gray areas between the stereotypes.

### **T helper 1 Cells**

The Th1 path is chosen when T cells are exposed to IL-12 during priming. Th1 cells are characterized by the production of the cytokine, interferon- $\gamma$  (IFN $\gamma$ ) and the expression of the master transcription factor, T-bet. Th1 cells are experts at gearing the immune response towards the control of internal pathogens like viruses and mycobacteria, which reside internally in macrophages. They perform this function by initiating cytotoxic T cell responses, helping macrophages to become more effective, by helping B cells to produce certain types of antibodies.

These functions are executed, in part, through IFN $\gamma$  exposure, however, some require cell-cell contact and will be explained in more detail later.<sup>37</sup>

### **T helper 2 Cells**

Th2 cells are created during exposure to high amounts of IL-4. This leads to the expression of the Th2-associated master transcription factor, GATA3. Th2 cells are also characterized by the production of IL-4 (indeed, the same cytokine needed to create them). These cells are designed to skew the immune system towards a humoral immune response (antibody response) that can deal with parasite infection. Unfortunately, Th2 responses are also the ones associated with allergy development as well. Th2 cells do their work by effectively helping B cells and encouraging specific forms of antibodies. This is done through a combination of IL-4 exposure and cell-cell interactions.

### **T helper 17 Cells**

The Th17 subtype is the most recently described of the Th subtypes. It is most effective at controlling extracellular bacterial and fungi responses, like those found during intestinal food poisoning or during a yeast infection. Its creation is dictated by the cytokines IL-6 and TGF $\beta$  and this leads to the expression of the master transcription factor, ROR $\gamma$ t. Th17 cells produce the cytokine IL-17. IL-17 production is one of the main facilitators of their function and it encourages surrounding cells to increase neutrophil migration. Neutrophils are excellent phagocytic cells with many bacterial killing tools.

### **Induced Regulatory T cells**

To those just learning about the immune system, the existence of the following Th subtype may be confusing. iTreg are designed to counter the functions of other immune cells. Why? The reason is that immune responses are highly

damaging to surrounding tissues and, without them, immune responses would spiral out of control.

That said; these cells are induced by DCs when they are exposed to high amounts of IL-10 or TGF $\beta$ . This causes the expression of the master transcription factor, Foxp3. In turn, iTreg produce IL-10 or TGF $\beta$ . IL-10 and TGF $\beta$  are what is called —anti-inflammatory cytokines. They have the ability to limit the functions of immune cells. IL-10, for instance, lowers Th1 and Th17 responses and reduces macrophage efficacy. TGF $\beta$  encourages apoptosis (induced death of cells), prevents cell division and lowers phagocytosis

### **Cytotoxic T cell Responses**

Th cells are not the only kind of T cell. Cytotoxic T cells (CTLs), characterized by the surface marker CD8, are not to be missed and are essential for the elimination of viral infections. The function of a CTL is found in its name. —Cytol refers to cell and —toxic means just how it sounds. These cells are —cell toxic and kill other cells. In many ways, they are similar to the NK cells and NK T cells of the innate immune system. However, they do not use invariant receptors to recognize problems in other cells, but instead use an adaptive system.

CTLs, like Th cells, have a TCR. This means that they can detect unique peptides presented to them by other cells. In the case of Th cells, these are MHC class II molecules presented via DCs. In the case of CTLs, they are MHC class I molecules. During an infection, as we earlier mentioned, DCs will travel to the lymph node and present samples of the intruder to the T cells. This also happens for CTLs. However, despite the presence of all the priming signals, priming will be suboptimal. CTLs need an additional signal, jokingly called —the license to kill.

This signal is given by a Th1 cell through the production of a cytokine called IL-2, which stimulates CTL expansion; and through an interaction between the Th1 cell and the DC via CD40 on the DC and CD40 ligand on the Th1 cell, which makes the DC more effective at priming CTLs<sup>33</sup>. Once a CTL is primed and active, it has the ability to kill.

As you can see, CTL activity is highly controlled to ensure that they react only to pathogen-associated peptides. The reason is that MHC class I can be expressed by every cell type in the body. MHC class I on a cell is like a sign advertising the health of the cell. The cell is constantly displaying samples of the proteins it's making. If an active CTL recognizes one of these samples as being of viral origin, it kills that cell; eliminating a viral host.

### **Adaptive Humoral Immune Responses**

The word - humoral means fluid in Latin and, therefore, humoral immune responses relate to non-cellular systems found in the bodily fluids. We've already discussed non-cellular components of the innate immunity, however, in immunology most people are not referring to these non - cellular systems when they use the term - humoral immune response. Instead, they are referring to the immune response mediated by antibodies and this is part of the adaptive immune response.

The cell behind antibody responses is the B cell. Naïve B cells of the immune system produce rudimentary antibodies (see below) until other cells activate them. B cells, unlike the T cells, are not required to interact with DCs; instead B cells reside in lymphoid tissues and fish for antigens that they recognize using their B cell receptors or BCR. The BCR looks like a surface bound antibody and once it binds a molecule, the B cell engulfs it and much like the phagocytes, digests it. Just like the DC, the B cell will then present pieces of the antigen to Th cells using MHC

class II molecules. Primed and activated Th cells, which recognize the presented peptides, are then able to —help the B cell through a CD40-CD40 ligand interaction. The Th cell also provides cytokine signals to tell the B cell which kinds of antibodies it should make.

This process is reminiscent of the priming process of Th cells. Signal one is the MHC class II/peptide and TCR interaction between the B cell and the T cell. Signal two is the costimulatory help provided by the T cell in the form of CD40-CD40 ligand interactions. And, signal three is the cytokine message provided by the T cell. Helped B cells will then further differentiate into plasma cells, which can produce massive quantities of antibodies.

### **Antibodies**

Antibodies, by themselves, cause very little harm. However, their strength lies in their ability to tag a molecule as harmful and block molecular functions. Antibodies enhance the functions of the innate immune system. They can bind to pathogens and particles to initiate the complement system and induce phagocytosis. They can also block/neutralize molecular interactions. Examples of this function would be an antibody that blocks the toxic effects of diphtheria toxin or antibodies that block viral binding sites to cells. Antibodies also interact directly with cells and can change their function by binding to specific antibody receptors found on the surfaces of immune cells.

An Antibody is a small protein structure produced by B cells. It is also called an immunoglobulin (Ig). It looks like a - Y and it is formed from four separate proteins. Each tip of the - Y recognizes and sticks to the antigen, meaning that each antibody can bind two similar antigens. A single arm is called a Fab (Fragment, antigen binding) fragment. The base of the - Y is called the Fc (Fragment constant)

region and, while the Fab fragments dictate the specificity of the antigen binding, the Fc region dictates the type of antibody or isotype. The antibody isotype is dictated by the prevalent cytokines in the environment as well as additional danger signals that the B cell experienced while being helped by the Th cell.<sup>41</sup>

### **Rudimentary Antibodies: IgM and IgD**

The first types of antibodies that a B cell can produce are IgM and IgD. The - M and - D refers to different classes of the Fc region. IgM is found as a pentamer, with five individual IgM antibodies bound by their Fc regions in the center forming a star. They are effective at complement activation. IgD is found as a monomer and its function is undefined. However, it has the ability to bind mast cells via an Fc $\delta$  receptor ( $\delta$  for D) and induce anti-microbial peptide secretion.

### **IgG**

IgG antibodies are found as monomers and they are very potent at stimulating immune responses. They are capable of neutralization, inducing phagocytosis in macrophages and neutrophils via Fc $\gamma$  receptors ( $\gamma$  for G), activation of complement, and also the activation of NK cells (also via Fc $\gamma$  receptors).

### **IgE**

IgE antibodies are monomers. They are known to cause mast cell degranulation via binding of Fc $\epsilon$  receptors ( $\epsilon$  for E). They are induced during parasite infection and, unfortunately, also during allergy.

### **IgA**

IgA is found as a dimer of two antibodies attached via their Fc regions. It is involved with mucosal defense: found in gastrointestinal system, the respiratory systems. They are particularly effective at neutralization of microbes and toxins. How the Adaptive Response Strengthens the Innate Response Once the adaptive

immune system has formed a response, the body has a long-term record of the invading pathogen in the form of long-lived plasma cells, memory T cells (not covered here) and antibodies. This is why vaccination is so important. It allows your body to create an adaptive immune response against an invader without having to truly become infected.<sup>42</sup> When a body encounters a pathogen for the second time, it's a completely different situation than the first encounter. During a second infection, T cells drawn to the inflammation site will have knowledge to help macrophages, recruit more neutrophils, and kill infected cells. Antibodies will be now present to assist complement activation, the phagocytosis of particles, and even kill microbes. The response will be quicker and more effective.

Though separating the two types of responses: innate and adaptive, helps with learning; it can also become an obstacle to seeing the immune response as a complex, dynamic system. It is important when looking at an immunological problem to consider the host's previous history as it has so much influence on the immune response.

## **1.6 Cytokines**

Cytokines are a group of low molecular weight regulatory protein secreted by leukocytes and a variety of other cells, in response to a number of inducing stimuli. Cytokines as general act as immune "messenger molecules" that modulate, educate, stimulate, and regulate various aspects of the immune response by acting on cells. Cytokines bind to specific receptors on the surface of target cells. Cytokines that are secreted from lymphocytes are termed lymphokines, whereas those secreted by monocytes or macrophages are termed monokines. Many of the lymphokines are also known as interleukins (ILs), since they are not only secreted by leukocytes but

also affect on leukocytes. Cytokines include interferons, stimulating factors, or necrosis factor. Some properties of cytokines are given below (**Kuby, 1994**).

Cytokines have been reported to be involved in the immunopathology of several autoimmune diseases including Type 1 diabetes (**Dinarello and Mier, 1987**). There is evidence that cytokines could have a direct role in N-cell death (**Mandrup- Poulsen *et al.*, 1986**). The macrophage released cytokines, TNF- $\alpha$  and IL-1, are cytotoxic to islet N-cells in vitro (**Mandrup-Poulsen *et al.*, 1986, Campbell *et al.*, 1988**). Interleukin-6, also produced by macrophages, is a key mediator of multiple inflammatory and immune responses and regulates insulin secretion in vitro in concert with IL-1 (**Campbell *et al.*, 1988**). IFN- $\gamma$ , produced by activated T lymphocytes, activates macrophages, enhances class I Major Histocompatibility Complex (MHC) antigen expression and induces class II expression in combination with TNF on normal cultured human islet cells (Pujol-Borrell *et al.*, 1987); IFN- $\gamma$  also enhances TNF induced human islet cells cytotoxicity (**Soldevila *et al.*, 1991**). These studies indicate that cytokines may have a role in the pathogenesis of Type 1 diabetes. The biological properties of these cytokines and their potential role in the pathogenesis of Type 1 diabetes will be discussed next

### **Cytokine antagonists**

There are several proteins that can inhibit the activity of cytokines. These proteins act by two ways. Either the antagonist can bind to the receptor without activating it, or to the cytokines directly preventing their further binding to the receptors. The best characterized inhibitor is the IL-1 receptor antagonist (IL-1Ra) which binds to the IL-1 receptor (IL-1R) without activation, and blocks it from



binding IL-1  $\alpha$  or  $\beta$  (**Dinarello, 1991**). The second group of the inhibitors is the soluble cytokine receptors that binds to cytokine and neutralize their activity. IL-2, IL-4, IL-6, IL-7, INF- $\gamma$ , TNF- $\alpha$ , TNF-13, and (Leukemia inhibitory factor) are among the soluble cytokine receptors that have been detected (**Foxwell, 1992**).

### **Interleukin-2 (IL-2)**

Interleukin 2 (IL-2) was one of the first well-characterized interleukin. Initially it was called T cell growth factor (TCGF), and its activity was detected in the supernatant of mitogen-stimulated peripheral blood lymphocytes (Theze, 1998). It is produced and secreted by activated T helper cells (CD4+), as the major interleukin responsible for proliferation and differentiation of T-cell and B-cell (Nakagawa *et al.*, 1985 and Muraguchi *et al.*, 1984). IL-2 alone induced the proliferation of T-helper 2 (Th-2) cells, which produce predominantly IL-4 and IL-5 and generate IgG1- and IgE-secreting cells as well as eosinophilia (Romagnani, 1992). Bogen and his colleagues (1993) observed IL-2 as one of the first cytokines produced in draining lymph nodes several days after immunization which supports the important role of IL-2 in initiating T-cell activation in lymphoid tissues.

### **Interleukin-6 (IL-6)**

Interleukin-6 is an interleukin that acts as both a pro-inflammatory and anti-inflammatory cytokine. In humans, it is encoded by the IL6 gene (Ferguson-Smith *et al.*, 1988). IL-6 is secreted by T cells and macrophages to stimulate immune response, e.g. during infection and after trauma, especially burns or other tissue damage leading to inflammation. IL-6 also plays a role in fighting infection, as IL-6 has been shown in mice to be required for resistance against bacterium *Streptococcus pneumoniae* (**van der Poll *et al.*, 1997**).

**Clinical use:** IL-6 is one of the most important mediators of fever and of the acute phase response. It is capable of crossing the blood and brain barrier (Banks *et al.*, 1994) and initiating synthesis of PGE<sub>2</sub> in the hypothalamus, thereby changing the body's temperature set point. In muscle and fatty tissue, IL-6 stimulates energy mobilization which leads to increased body temperature. IL-6 can be secreted by macrophages in response to specific microbial molecules, referred to as pathogen associated molecular patterns (PAMPs). These PAMPs bind to highly important group of detection molecules of the innate immune system, called pattern recognition receptors (PRRs), including Toll-like receptors (TLRs). These are present on the cell surface and intracellular compartments and induce intracellular signaling cascades that give rise to inflammatory cytokine production (Bastard *et al.*, 1999). IL-6 is relevant to many diseases such as diabetes (**Kristiansen *et al.*, 2005**) atherosclerosis (Dubínski *et al.*, 2007) depression (Dowlati *et al.*, 2010) Alzheimer's Disease, (Swardfager *et al.*, 2010) systemic lupus erythematosus (**Tackey *et al.*, 2004**) prostate cancer (**Smith *et al.*, 2001**) and rheumatoid arthritis (**Nishimoto, 2006**).

### 1.8 Immunomodulation

Immunomodulation is a very broad term which denotes to any changes in the immune response and may involve induction, expression, amplification or inhibition of any part or phase in the immune response (**Sell, 1987**). Modulation of the immune response may involve induction, expression, amplification, or inhibition of the afferent, central, efferent, or accessory phase of the immune response. Immunomodulation may be specific or nonspecific (**Stewart, 1987**). The immunomodulating drugs are needed for the treatment of various disease statuses

such as infections, organ transplantation, cancer, rheumatoid arthritis, systemic lupus erythematosus, Down syndrome, Crohn's and autoimmune diseases and the acquired immune deficiency syndrome (AIDS).

Immunomodulation is the regulation and modulation of immunity either by enhancing or by reducing the immune response. Modulation of immune response may involve induction, expression or amplification of immune response. In other words, immunomodulation involves a change in the human body's immune system caused by agents that activate or suppress its function. If the modulation in immune system results in enhancement of immune reaction, it is known as the immunostimulation. There are two main categories of immunostimulators. The specific immunostimulators are those which provide antigenic specificity in immune response, such as vaccines or any antigen; the non-specific immunostimulators are those which act irrespective of antigenic specificity to augment immune response of other antigens or stimulate components of the immune system without antigenic specificity, such as adjuvants and non-specific immunostimulators (**Sunil *et al.*, 2011**).

Most significant progress in the field of immunomodulators is represented by the discovery of cyclosporin. It is a potent immunosuppressant that has proved to be a boon for prevention of graft rejection. The drug is also gaining ground for treatment of several autoimmune diseases, particularly in failures of prednisolone, azathioprin, cyclophosphamide and methotrexate. However, due to its very low therapeutic index and significant nephrotoxicity, search for an alternative to cyclosporine is being actively pursued (**Walsh *et al.*, 1992**)

**Immunoadjuvants**

These agents are used for enhancing vaccines efficacy and therefore, could be considered specific immune stimulants. An example in this regard is of Freund's adjuvant. The immunoadjuvants hold the promise of being the true modulators of immune response. It has proposed to exploit them for selecting between cellular and humoral, Th1 (helper T1 cells) and Th2, (helper T2 cells) immunoprotective and immunodestructive, and reagenic (IgE) versus immunoglobulin G (IgG) type of immune responses, which poses to be a real challenge to vaccine designers.

**Immunostimulation**

These agents are envisaged to enhance body's resistance against infections (and may be against allergy, autoimmunity, and cancer as well). By this definition these agents are inherently non-specific in nature, but they can act through both the innate and adaptive arms of the immune response. In healthy individuals the immunostimulant are expected to serve as prophylactic agent i.e. as immune potentiates by enhancing the basal levels of immune response, and in individuals with impairment of immune response as immunotherapeutic agent. The immune compromised conditions include patients with primary (humoral, cellular or combined immune deficiency syndromes) as well as secondary immune deficiencies (AIDS, malignancy, cancer chemotherapy, patients receiving steroids etc). Considering that these agents may not be effective by themselves, they may be used as adjunct to chemotherapy to remove residual cancer cells, as well as in treatment of chronic/persistent/latent infections (viral, parasitic etc) with or without available chemotherapeutic agents.

Increasing urgent need to develop new effective herbal remedies or drugs for health care; traditional medicinal plants have recently received adequate attention of the western countries, pharmaceutical companies and scientific communities (Planning Commission, Govt. of India, 2000; Alam *et al.*, 2004; Ladjel *et al.*, 2011). Herbal formulations are effective, relatively cheaper and safe alternative treatment for various diseases since most of the synthetic drugs available in the market have more side effects and provide only symptomatic relief (Fulzele *et al.*, 2002). The present scenario thus forces hard to discover newer drugs from our broad biodiversity rich reservoir of plant kingdom which may provide therapeutic cure and would be free from undesirable side effects as well as economical, which would easily be accepted by the developing nations.

Many of the plant products exert their effect through immune system; hence, a number of plant species, to days, are being investigated for their products in development of immune response modifiers or Immunomodulators (Upadhyay, 1997). Treatment and prevention of infectious diseases are the most common reasons to use immunomodulators. These sorts of agents are becoming very popular in the worldwide natural health industry as people have started realizing the importance of a healthy immune system in maintenance of health and prevention and recovery of disease since immunomodulators do not tend to boost immunity but to normalize it (Sehar *et al.*, 2008; Agrawal *et al.*, 2010). The mode of action of immunomodulators in the body is still largely a mystery, however, a part of their beneficial effects appears to be because of their ability to naturally increase the production of cytokines, which mediate and regulate the immune system. The primary target of most of the immunomodulatory compounds are believed to be

macrophages, which play a key role in the generation of immune response. Activated macrophages produce a number of intermediates of reactive oxygen species (ROI/ ROS) and nitric oxide (NO) that have antimicrobial activity (Sharp *et al.*, 1993; Drapier, 1997; Syamsudin *et al.*, 2008). Continued discovery of new immune regulators and increased understanding of immunity will ensure newer opportunities for the use of immunomodulators in medical science.

Immunomodulators have the ability to mount an immune response or defend against pathogens or tumors and can safely be used to alleviate hyper- or hypo-immune responses or against various diseases that accompany immune suppression viz. Acquired Immune Deficiency Syndrome (AIDS), Leishmaniasis, Filariasis, Tuberculosis and Malaria (Wagner, 1990; Dwivedi *et al.*, 2008; Samant *et al.*, 2009; Singh *et al.*, 2009; Mahiuddin *et al.*, 2010; Patel *et al.*, 2010). Immunomodulators may also serve as immunological adjuvants to presently available standard drugs or vaccines to boost their efficacy (Mahiuddin *et al.*, 2010). The capacity of adjuvants to activate antigen presenting cells (APCs) during induction of primary immune response is of critical importance for development of protective immunity against a number of pathogens which can be cured by these immune modifiers when used prophylactically.

In spite of the availability of drugs against several diseases newer agents are required to fulfill drawbacks of the currently used drug and to combat drug resistance commonly encountered or fast emerging against some pathogens. A number of plants have been identified to possess immunomodulatory or therapeutic efficacy and even active chemical constituents have also been identified, however, many still remain unexploited and need thorough investigation viz. *Annona*

*squamosa* (AS), *Murraya koenigii* (MK), *Withania coagulans* (WC) and some chemo-types of *Withania somnifera* (WS) which are widely consumed by human population in India.

*Annona squamosa* L. (Family: Annonaceae), commonly known as Custard Apple or Sugar Apple or 'Shareefa' is a native of West Indies and is cultivated throughout India, mainly for its edible fruit. The plant is attributed with several medicinal properties which include antifertility and anti-tumour activities in mice and rats (Rao *et al.*, 1979; Asolkar *et al.*, 1992; Yang *et al.*, 2008). The fruit pulp due to its richness in free sugars, minerals and vitamins is known to serve as blood tonic (Rao, 1974). The young leaves of AS are used extensively for its antidiabetic activity by tribal men in and around the villages of Aligarh district in the state of Uttar Pradesh, India (Atique *et al.*, 1985) and also by the people of Chota Nagpur district in the state of Bihar, India (Topno, 1997).

*Murraya koenigii* (Family: Rutaceae), commonly known as Curry-leaf tree is found almost everywhere in the Indian subcontinent, excluding the higher levels of the Himalayas (Rastogi and Mehrotra, 1998; Shah *et al.*, 2008). MK is widely used as a spice and condiment in India and other tropical countries. The leaves, bark and the root of this plant are used intensively in indigenous medicine from ancient time, as a tonic for stomachache, stimulant and carminative (Pruthi, 1998). MK leaves mixed with fat separated butter is used for the treatment of amoebiasis, diabetes and hepatitis in Ayurveda (Pillai and Gopala, 1958; Bose and Chandra, 1985; Satyavati *et al.*, 1987) and it is traditionally consumed by diabetics in southern part of India (Yadav *et al.*, 2002).

*Withania coagulans* Dunal (Family: Solanaceae) is a shrub commonly known as Indian cheese maker occurs in dry parts of India. Traditionally the plant is used for the control of diabetes mellitus, dyspepsia, flatulent colic and for diuretic purpose (Kirthikar and Basu, 1933; Hemalatha *et al.*, 2008). The fruits of this plant are used for coagulation of milk and as blood purifier (Ali *et al.*, 2009). Different parts of this plant have been reported to possess a variety of biological activities viz. antibacterial (**Budhiraja *et al.*, 1987; Khan *et al.*, 1993**), antifungal (Choudhary *et al.*, 1995), anti-inflammatory (Budhiraja *et al.*, 1984), Cardiovascular (Hemalatha *et al.*, 2008) and antitumor activity (Chattopadhyay *et al.*, 2007). The plant was also reported to have hepatoprotective, antihyperglycaemic, hypolipidaemic, free radical scavenging, antimicrobial, central nervous system depressant, antitumour and cytotoxic activities (**Maurya, 2010**).

*Withania somnifera* (Family: Solanaceae) is a shrubby xerophytic herb, commonly known as Winter cherry or Ashwagandha. It is cultivated in India, East Asia and Africa and can also grow at high altitude up to 5,500 feet in the Himalayas (Uddin *et al.*, 2012). It offers tremendous medicinal properties including antibacterial, anti-inflammatory, anti-fungal, antitumor and immune-boosting (immunomodulatory) properties (Singh *et al.*, 2010). It has also been reported to have beneficial effects in several cases including rheumatoid arthritis, polyarthritis, lumbago, painful swellings, spermatorrhoea, asthma, leucoderma, general debility, sexual debility, amnesia, anxiety neurosis (Uddin *et al.*, 2012). It helps improve vitality and combat insomnia, cancer, arthritis and diabetes. Although its immunomodulatory properties are well known, however, its chemotypes still need further exploration (**Gupta *et al.*, 2003**).



### **Aims of Immunostimulation**

Immunostimulation constitutes an attractive alternative to conventional chemotherapy and prophylaxis of infections, especially when the host defense mechanisms have to be activated under conditions of impaired immune responsiveness (Wagner *et al.*, 1985a). Immunostimulants or immunopotentiators are compounds leading predominantly to a nonspecific stimulation of the immunological defense system. They do not affect immunological memory cells. Therefore the terms immunomodulation or immunoregulation, denoting any effect on or change of immune responsiveness, very often seem to be more appropriate. Immunoadjuvants are substances that enhance the production of antibodies without acting as antigens themselves. Their effects are often thymus-dependent

### **Mechanism of Immunostimulation**

Immunological defence is a complicated interplay between nonspecific and specific, cellular and humoral immune responses, stimulation and suppression of immune competent cells, and the influence of endocrine and other mechanisms upon the immune system. Primary targets of the immunostimulant are T or B lymphocytes or the complement system, an increase in phagocytosis by macrophages and granulocytes plays a central role in immunostimulation (**Kuby, 1994**). Activation of macrophages is probably important for the stimulating agents to remain in contact with the reactive cell. The second most important role is the stimulation of T lymphocytes, which can be achieved either directly or indirectly, via macrophages (**Wagner *et al.*, 1985**)

**DNA THERAPY**

An alternative method of DNA-based immunization that has received significant attention recently is the use of CpG-rich (cytosine phosphorothioate-linked guanosine DNA) immunostimulatory DNA sequences as inhibitors of Th2 responses to antigen. Interest in this approach began when studies demonstrated that the CpG DNA motifs in *Mycobacterium bovis* BCG DNA induced interferon- $\gamma$ , a cytokine produced by Th1 cells.<sup>56</sup> In contrast, the immunostimulatory effect of vertebrate DNA is significantly lower than that of bacterial DNA. The reduced immunostimulatory effect of vertebrate DNA is probably related to a combination of the lower frequency of CpG DNA motifs in vertebrate compared to bacterial DNA, as well as a high frequency of cytosine methylation in vertebrate compared to bacterial DNA, which abolishes the immunostimulatory effect of vertebrate CpG DNA sequences. The DNA hexamer sequences generating the optimal ThL adjuvant effect in mice include the motif 5' - purine-purine-CpG-pyrimidine-pyrimidine-3', e.g. AACGTT or GACGTC Which activates the secretion of IFN- $\gamma$ . CpG DNA has an indirect effect on the adaptive immune response by activating the innate immune system (e.g., plasmacytoid dendritic cells) to upregulate cytokine expression (IL-12, IL-18, IFN- $\gamma$ , IFN- $\alpha$ , IFN- $\beta$ ), upregulate MHC molecules, and upregulate co-stimulatory molecules. These effects on the innate immune system lead to a cytokine milieu (IFN- $\gamma$ +, IL-12+) which biases the T-lymphocyte immune response towards a Th1 response to newly encountered antigens.

Studies have demonstrated an important role for a Toll receptor TLR9 (Toll receptor 9)<sup>58</sup> in mediating signaling of CpG DNA, as the deletion of genes encoding TLR-9 in mice results in mice that are unable to respond to CpG DNA.<sup>58</sup> As TLR9

in humans is expressed primarily by plasmacytoid dendritic cells and B cells, CpG DNA exerts its immunomodulatory effects in humans predominantly through its effects on plasmacytoid dendritic cells, which subsequently influence the adaptive immune response. Toll-like receptors (expressed on a variety of innate immune cells) belong to a family of pattern recognition receptors that are postulated to allow the innate immune system to sense different classes of pathogen.

### **ANTISENSE OLIGODEOXYNUCLEOTIDE THERAPY**

The goal of antisense oligodeoxynucleotide (ODN) therapy is to selectively inhibit the expression of a specific gene product by preventing the translation of mRNA into protein<sup>68</sup> (Fig. 94.6). Antisense ODN therapy acts by sequence-specific hybridization to mRNA, inhibiting the expression of that specific gene product while not affecting the expression of other genes. Antisense ODN therapy prevents the translation of the RNA message into protein and promotes the degradation of the message by ribonucleases. In order for antisense ODNs to be synthesized the coding sequence of the gene to be inhibited must be known. Antisense compounds are generally about 20 base pairs of oligodeoxynucleotides that have a sequence complementary to a portion of the targeted mRNA. The poor stability of oligonucleotides in vivo has been improved by modifying the phosphodiester backbone to a sulfur-containing phosphorothioate backbone, which enhances the stability of oligonucleotides to resist enzymatic degradation.

### **CYTOKINE-BASED THERAPY**

Cytokines play a key role in regulating the initiation, perpetuation, and resolution of allergic inflammation (Chapter 10). Based on studies demonstrating

that several cytokines are expressed in the airway in asthmatics, novel therapeutics have been developed to target individual cytokines in patients with asthma to determine whether any of these individual cytokines may play an important role in disease pathogenesis.

Pre-clinical studies in animal models of asthma have demonstrated that targeting individual cytokines such as TNF, IL-4, or IL-5 reduces levels of eosinophilic airway inflammation and airway responsiveness.

## **TNF**

To date, the principle that one can target a single cytokine and have a significant impact on human disease expression has been best validated in the treatment of autoimmune disease such as rheumatoid arthritis (RA) with inhibitors of the cytokine TNF- $\alpha$ .<sup>79</sup> Currently there are three TNF inhibitors used in clinical practice: infliximab (a chimeric monoclonal antibody), etanercept (a soluble TNF receptor/Fc fusion protein), and adalimumab (a human monoclonal antibody).<sup>79</sup> TNF inhibitors have also been shown to be highly effective in treating a number of other systemic inflammatory autoimmune diseases, including ankylosing spondylitis, psoriatic arthritis, psoriasis, and Crohn's disease. The success of TNF- $\alpha$  inhibitors in RA represents a proof-of-concept that inhibition of a single cytokine can be effective in treating a disease in which multiple cytokines are expressed, if that cytokine serves a key or central role. Most RA patients respond to TNF- $\alpha$  inhibitors with a reduction in signs and symptoms, improved quality of life, and preservation of functional status.<sup>79</sup> Radiographs demonstrate objective evidence that TNF- $\alpha$  inhibitors also significantly slow disease progression in RA to an extent not seen with any previous agents. Interestingly, although initial experience with

TNF inhibitors was most commonly in RA patients with chronic refractory disease, more recent studies have demonstrated even better clinical outcomes when TNF inhibitors were used earlier in the disease course.<sup>80</sup> As TNF inhibitors do not induce immunologic tolerance, maintenance of clinical efficacy almost always requires continued therapy, certainly for patients with long-standing RA. Importantly, 2–4-year and longer studies of anti-TNF- $\alpha$  therapy in RA suggest that the clinical response is well maintained. Nevertheless, some patients fail to respond to anti-TNF therapy and others eventually lose their response. Interestingly, patients failing one TNF inhibitor may respond to therapy with another.

Inhibition of TNF has also been studied in patients with asthma,<sup>81–84</sup> although results have not been as clear cut nor as dramatic in all asthma studies as in autoimmune conditions. Examples of studies in asthma which have shown a benefit to TNF blockade include studies of the soluble TNF receptor etanercept in 10 patients with severe asthma, which noted improvements in FEV<sub>1</sub>, airway responsiveness to methacholine, and asthma-related quality of life.<sup>81</sup> In another study of 38 symptomatic subjects with moderate asthma, infliximab did not demonstrate significant clinical efficacy in terms of the primary endpoint of lung function. However, there was a significant reduction in the number of moderate asthma exacerbations in the infliximab group compared to placebo. It is possible that only a subset of severe asthmatics respond to anti-TNF therapy, and that levels of expression of membrane-bound TNF- $\alpha$  by peripheral blood monocytes in such subjects with asthma may predict responses.

**IL-4**

IL-4 mediates several important proinflammatory functions in allergic inflammation, including induction of the IgE isotype switch, induction of vascular cell adhesion molecule-1, promotion of eosinophil transmigration across the endothelium, stimulation of mucus production, and promotion of Th2 lymphocyte differentiation. The therapeutic potential of a recombinant soluble IL-4 receptor (IL-4R) as an IL-4 antagonist has been studied in asthmatics. In two small studies, treatment with the IL-4 receptor antagonist improved asthma symptom scores and pulmonary function, reduced  $\beta_2$ -agonist rescue use, as well as lowering levels of exhaled nitric oxide. However, subsequently two large phase III studies in moderate-to-severe asthma failed to reveal efficacy, possibly due to dose limitations and the short duration of action of the IL-4 receptor antagonist.

**IL-5**

As IL-5 is a key regulator of eosinophil proliferation, studies have investigated whether targeting IL-5 would reduce eosinophilic inflammation and improve asthma outcomes. In asthmatics, therapy with an anti-IL-5

**Immunosuppression**

These agents could be used for control of pathological immune response in autoimmune diseases, graft rejection, graft versus host disease, hypersensitivity immune reaction (immediate or delayed type), and immune pathology associated with infections. Out of the list the maximum use of these agents has been for prevention of graft rejection and treatment of autoimmune diseases

**Physical immunosuppression Methods**

Surgical manipulation can have a major impact on immune responsiveness. Surgical removal of the bursa, or the thymus, or both in the neonatal period block the development of immunologic competence in the correspondingly dependent lymphoid system. Surgical removal of these tissues after immunologic development, however, has very little effect on immune competence.

**Radiation:** acts on the lymphoid cells and bone marrow. Radiation damages DNA; thus, cells that are in the process of division or that need to divide to express their immunologic role will be most affected by this exposure. It is used in the treatment of lymphoid malignancies (i.e. Hodgkins disease) and has been attempted in other T-suppressor cell autoimmune disorders. The most common adverse effects of TLI are moderate constitutional symptoms including fatigue, anorexia, diarrhea, abdominal pain, nausea, weight loss, xerostomia, and herpes zoster (**Pamphilon *et al.*, 1991**)

**Pheresis:** Pheresis is the discriminating removal of a specific blood component (e.g., platelets, plasma, red blood cells, leukocytes, and lymphocytes). Within hours to days, plasmapheresis removes antibodies, immune complexes, hormones, drugs, and other plasma-soluble substances. Plasmapheresis is used in the following immunologically mediated disorders: hyperviscosity syndromes associated with myelomas, mushroom poisoning, theophylline toxicity, Goodpastures syndrome, autoimmune hemolytic anemia, myasthenia gravis, and systemic lupus erythematosus (Hughes *et al.*, 1997). Plasma exchange is the process of separating and removing plasma, then replacing it with either fresh frozen plasma or plasma protein fractions. Selective forms of cellular pheresis are used to treat a variety of

disorders. For example, plateletpheresis is used for essential thrombocytosis, hemapheresis for sickle cell anemia, and lymphocytapheresis for leukemias (Rosenberg *et al.*, 1986).

**Blood transfusion:** Transfusions have also altered immune function by either improving renal allograft survival or decreasing bone marrow transplant engraftment survivals (Blumberg *et al.*, 1990).

**1.9 Immunosuppressive Drug Cyclophosphamide:** Cyclophosphamide also known as cytophosphane, is a nitrogen mustard alkylating agent from the oxazophorines group. Cyclophosphamide has been shown to suppress primary and secondary humoral immune response, delayed type of hypersensitivity, skin graft rejection and animal diseases of autoimmunity (Eric R. Hurd, 1973). It is used to treat various types of cancer and some autoimmune disorders. A prodrug, it is converted in the liver to active forms that have chemotherapeutic activity. Cyclophosphamide also decreases the immune system's response to various diseases and conditions. Therefore, it has been used in various non-neoplastic autoimmune diseases where disease-modifying antirheumatic drugs (DMARDs) have been ineffective (Steinberg *et al.*, 1971). It is also used to treat minimal change disease, severe rheumatoid arthritis (Townes *et al.*, 1976), Wegener's granulomatosis (Novack and Pearson, 1971), and multiple sclerosis (Makhani *et al.*, 2009).

Recent clinical studies have shown cyclophosphamide induces beneficial immunomodulatory effects in the context of adaptive immunotherapy. Although the mechanisms underlying these effects are not fully understood, several mechanisms have been suggested based on potential modulation of the host environment, including



1. Elimination of T regulatory cells (CD4+CD25+T cells) in naive and tumor-bearing hosts
2. Induction of T cell growth factors, such as type I IFNs, and
3. Enhanced grafting of adoptively transferred tumor-reactive effectors T cells by the creation of an immunologic space niche.

Thus, cyclophosphamide preconditioning of recipient hosts (for donor T cells) has been used to enhance immunity in naïve hosts, and to enhance adoptive T cell immunotherapy regimens, as well as active vaccination strategies, inducing objective antitumor immunity. Arnold and Bourseaux (1958) first synthesized Cyclophosphamide, which interferes with cell growth only after activation in tissue. It is capable of inhibiting both humoral and cell-mediated immune response (Shand *et al.*, 1979, Doherty, 1981).

**Azathioprine:**

*Azathioprine* (IMURAN) is a purine antimetabolite which is used as a first-line therapy or as an alternative to nitrogen mustard, alkylating agents, for treatment of immune-mediated disease. Azathioprine, an imidazole derivative of 6-mercaptopurine, that acts as an Antimetabolite to inhibit nucleic acid synthesis via both *de novo* and salvage pathways. Rapidly dividing cells, including not only T- and B-cell lymphocytes but also, for example, gut endothelium and bone marrow elements, are most susceptible to the drug's antiproliferative effects. In addition, it has been suggested that the imidazole residue of Azathioprine alkylates thiol groups on T-cell surface membranes block antigen recognition. Myelosuppression, particularly leukopenia, is the most common and serious side effect of Azathioprine therapy (Rossi *et al.*, 1993). It induced hepatotoxicity due to endothelial damage and may cause veno-occlusive disease (Sterneck *et al.*, 1991). Other toxicities relate to

its antimitotic effects, including alopecia, oral ulcers, nausea, vomiting, diarrhea, anorexia and esophagitis. The increased incidence of malignancies with Azathioprine tends to be less than that observed with other immunosuppressants, probably owing to its modest relative potency (Blohme and Brynger, *et al.*, 1985; Rossi *et al.*, 1993).

### **Methods for Testing Immunological Factors**

The routine process for screening is to extract single ingredient or single distilled fraction from herbal drugs, determine its bioactivity by the classic pharmacological means. The whole animal model is the most classic pharmacological screening model, which is very important at the aspect of medicine evaluation because it can apparently respond to the efficacy, side effect and toxicity of medicines in whole. Although this method is high cost and low efficient, at present it is still a primary way to drug discovery and evaluation

#### ***In vitro* methods:**

1. Inhibition of histamine release from mast cells
2. Mitogen induced lymphocyte proliferation
3. Inhibition of T cell proliferation
4. Chemiluminescence in macrophages
5. PFC (plaque forming colony) test *in vitro*
6. Inhibition of dihydro-orotate dehydrogenase

#### ***In vivo* methods:**

1. Spontaneous autoimmune diseases in animals
2. Acute systemic anaphylaxis in rats
3. Anti-anaphylactic activity (Schultz-Dale reaction)
4. Passive cutaneous anaphylaxis

5. Arthus type immediate hypersensitivity
6. Delayed type hypersensitivity
7. Reversed passive arthus reaction
8. Adjuvant arthritis in rats
9. Collagen type II induced arthritis in rats
10. Proteoglycan-induced progressive polyarthritis in mice
11. Experimental autoimmune thyroiditis
12. Cocksackievirus B3-induced myocarditis
13. Porcine cardiac myosin-induced autoimmune myocarditis in rats
14. Experimental allergic encephalomyelitis
15. Acute graft versus host disease (GVHD) in rats
16. Influence on SLE-like disorder in MRL/lpr mice
17. Prevention of experimentally induced myasthenia gravis in rats
18. Glomerulonephritis induced by antibasement membrane antibody in rats
19. Auto-immune uveitis in rats
20. Inhibition of allogenic transplant rejection

## **AUTOIMMUNITY**

Overactive immune responses comprise the other end of immune dysfunction, particularly the autoimmune disorders. Here, the immune system fails to properly distinguish between self and non-self, and attacks part of the body. Under normal circumstances, many T cells and antibodies react with “self” peptides. One of the functions of specialized cells (located in the thymus and bone marrow) is to present young lymphocytes with self antigens produced throughout the body and to eliminate those cells that recognize self-antigens, preventing autoimmunity.

**IMMUNOLOGIC EFFECTS**

Inhibition of cell replication may be the key therapeutic mechanism of methotrexate in neoplastic conditions. Lymphocytes divide rapidly in the course of the immune response, and thus immunosuppression may be one mechanism of action of methotrexate in various immunologic diseases. However, several observations have suggested that other mechanisms may be relevant in non-malignant conditions. For example, in contrast to its use in the treatment of malignancies, the lower doses of methotrexate commonly used in rheumatoid arthritis (RA) are infrequently associated with myelosuppression or clinical signs of systemic immunosuppression. In addition, supplementation with low doses of folic acid may obviate some of the toxicity seen in RA patients receiving methotrexate without significantly attenuating its clinical efficacy.<sup>1,2</sup> In vivo studies have shown that methotrexate can induce various immunomodulatory effects, including effects on cellular immunity, humoral immunity, and inflammation.<sup>1,3,4</sup> Two relevant mechanisms underlie the anti-inflammatory effect of methotrexate at the doses typically used in non-malignant conditions:

- (1) Inhibition of the intermediate step in purine metabolism catalyzed by amino-imidazole-carboxy-amido-ribonucleotide (AICAR) transformylase, leading to the increased release of adenosine; and
- (2) Interference with transmethylation reactions, such as the methylation of homocysteine to methionine.<sup>1</sup> The precise mechanisms by which AICAR inhibition results in increased extracellular concentrations of adenosine remain to be defined, but through interactions with specific receptors (A1, A2a, A2b, A3) on various cell types, adenosine exerts potent and diverse antiinflammatory actions.

**INTRAVENOUS IMMUNOGLOBULIN**

In healthy individuals, following IVIG infusion there is a biphasic plasma IgG disappearance curve, with the initial phase representing distribution between body compartments and early catabolism, and the second phase representing catabolism. The half-life of IVIG ranges from 14 to 24 days in healthy individuals and is more prolonged (26–35 days) in patients with humoral immunodeficiencies. IVIG has a variety of immunomodulating properties. Which are of potential benefit in immunologically mediated diseases such as immune thrombocytopenic purpura and Kawasaki syndrome. These immunologic effects include blockade of Fc receptors, anti-cytokine effects, down regulation of T- and B-cell function, inhibition of complement activation. enhanced clearance of endogenous IgG, anti-idiotypic suppression, and neutralization of super antigens.<sup>30</sup> Which, if any, of these effects may be useful in the treatment of asthma or allergy is currently not known. In vitro studies have shown that IVIG inhibits cytokine dependent lymphocyte proliferation, as well as cytokine (IL-2, IL-4) production by T lymphocytes.

**Immunoglobulin E**

Inhibition of IgE production by B cells is one postulated mechanism by which IVIG may exert an immunomodulating effect in asthma. This effect of IVIG is postulated to occur through co-ligation of the B cell Fcγ RIIB receptor and the B-cell antigen receptor, with resultant negative signaling in B cells. IVIG could thus provide an off signal to the B cell to inhibit B-cell proliferation and immunoglobulin production. In vitro, IVIG inhibits IgE production by B cells. In open-label studies IVIG has been shown to reduce the immediate skin reactivity to allergen. Although there is some experimental evidence to suggest that

IVIg may reduce IgE levels in vivo a reduction in IgE is not noted in the majority of studies which have investigated this immunomodulatory property of IVIg.

### **Sino pulmonary infections**

IVIg might theoretically improve asthma by reducing the incidence of sinopulmonary infections. Evidence for this has not adequately been addressed in current studies with IVIg.

### **Medicinal values for some of the sources of the used compound**

Few medicinal values of some selected plants are discussed below. *Buxus* Plant species are distributed throughout the world. More than 150 new steroidal alkaloids have been isolated from different *Buxus* species (Naz *et al.*, 1995). Extracts of various species have been used to treat rheumatism, malaria, depression, skin and venereal diseases in folk medicine (Cordell, 1981, Naz *et al.*, 1995). The crude ethanolic extract of the plant is reported to be active against the human immunodeficiency Virus (HIV) and other diseases in which the or necrosis factor is involved and it has also been reported in the literature that trail dose administration of *B. sempervirens* preparation (SPV30) in HIV-infected asymptomatic patients have shown a delay in the progression of HIV disease and no severe side effects were observed (Durant *et al.*, 1998). Cyclobuxine-D isolated from *B. microphylla* has shown a hypotensive, anti-inflammatory, and bradycardiac effects and found to protect rat heart from the myocardial injuries (Lee *et al.*, 1993). *Withania* plants e.g. *W. coagulants* and *W. somnifera* grow widely in India and Pakistan and used in indigenous system of medicine. *Withania somnifera* has been repeatedly shown to be a very potent immunomodulatory agent, apart from being anti-tumor, hepatoprotective (Ahumada *et al.*, 1991, Bhakuni *et al.*, 1969). Sitoindoside VII,

VIII, IX & X isolated from *W. somnifera* has already been proved for their immunomodulatory activities (Bhattacharya *et al.*, 1997). Withanoloids are very interesting since many of them show potent antitumor, antibacterial, antifungal, anti-inflammatory and immunosuppressive activity (Atta-ur-Rahman and Choudhary, 1999, Devi *et al.*, 1992).

Corchorus this genus contain about 100 species which are distributed mainly in south-East Asia and South America (Chopra *et al.*, 1956). *Corchorus deppressus* L is one of those grow in this region. Leaves are used in folk medicine as an emollient and cooling agent. The mucilage of these plants is used for treatment of gonorrhoea and applied as a poultice for healing wounds (Perry and Metzgar, 1980). The decoction of the seeds and leaves used as a tonic and the infusion is used as a drink to relief fever (Whaid and Siddique, 1961) as well it is found to have antipyretic antianalgesic activity (Ikram *et al.*, 1987, Vohora *et al.*, 1981). *Tephrosia purpurea* has been shown to possess significant activity against hepatotoxicity, pharmacological and physiological disorders. It inhibits benzoyl peroxide-mediated cutaneous oxidative stress and toxicity marine skin. Topical application of *Tephrosia purpurea* prior to each application of croton oil (phorbol ester) resulted in a significant protection against cutaneous carcinogenesis (Saleem *et al.*, 2001). Extracts from these plants have insecticidal, pesticidal, antihelminthic, anticancer, and antiulcer activity and are used in traditional medicines (Pirrung *et al.*, 1998). *Pteleopsis hyiodendron* plants of the genus pteleopsis are known to possess medical importance. It is used in African countries for treatment of gastric and duodenal ulcers (Germano *et al.*, 1998). *Vitex agnus castes* the Chaste tree berry extracts found to inhibit basal as well as TRH-stimulated prolactin secretion of rat pituitary

cells *in vitro* (Sliutz *et al.*, 1993). Most of the activities and medicinal uses of this plant is related to female menopause.

*Anogeissus sheimperi* this is a small genus of Combretaceae family comprising 5 species found in tropical Africa and Asia. Several triterpenoids were isolated including oleanolic acid from this plant (Atta-ur-rahman *et al.*, 2003). Oleanolic acid compound is quite common in nature and has now become a multifaceted of a number of pharmacological activities since it possesses anti-HIV, antiviral, anti-inflammatory, sedative, hepato-protective, analgesic, cardiogenic and tonic effects (Mengoni *et al.*, 2002, Liu *et al.*, 1995). It has been reported for inhibition of or promotion *in vitro* and *in vivo* experiments (Ohigashi *et al.*, 1986, Wang and Jiang, 1992) and in it is recommended for skin cancer therapy (Oguro *et al.*, 1998).

**Table. No. Plants having Immunomodulatory activity**

S.No.	Plant name	Family	Part use
1	<i>Boerhaavia diffusa</i>	Nyctaginaceae	Root
2	<i>Curcuma longa</i>	Zingiberaceae	Rhizome
3	<i>Rhododendron spiciferum</i>	Ericaceae	Leaf
4	<i>Caesalpinia bonducella</i>	Caesalpinaceae	Whole plant
5	<i>Tinospora cordifolia</i>	Menispermaceae	Whole plant
6	<i>Capparis zeylanica</i>	Capparidaceae	Whole plant
7	<i>Lufa cylindrica</i>	Cucurbitaceae	Seed and fruit (bulb)
8	<i>Withania somnifera</i>	Solanaceae	Whole plant
9	<i>Asparagus racemosus</i>	Asparagaceae	Root
10	<i>Panax ginseng</i>	Araliaceae	Root
11	<i>Nelumbo nucifera</i>	Nymphaeaceae	rhizome and seed
12	<i>Azadirachta indica</i>	Meliaceae	Leaf
13	<i>Arnica montana</i>	Compositae	Dried flowers head
14	<i>Calendula officinalis</i>	Asteraceae	Flower
15	<i>Echinacea purpurea</i>	Asteraceae	Flowering top
16	<i>Euphorbia tirucalli</i>	Euphorbiaceae	Latex
17	<i>Ocimum sanctum</i>	Lamiaceae	Leaf



## 2. AIM AND OBJECTIVE

### 3.1 General objective

- ✚ To evaluate *in vivo* immunomodulatory activity of ethanolic extract of *Annona reticulata*. Linn fruits.

### 3.2 Specific objective

- ✚ Extraction and characterization of component from ethanolic extracts of fruits of *Annona reticulata*. Linn.
- ✚ To evaluate acute toxicity study of ethanolic extracts of fruits of *Annona reticulata*. Linn
- ✚ To evaluate *in vivo* immunomodulatory activity of ethanolic extracts of fruits of *Annona reticulata*. Linn by following methods
  - Delayed type hypersensitivity (DTH) response
  - Humoral antibody (HA) titer
  - Total leukocyte count
  - Determination of total serum protein

### 3. PLAN OF WORK

- Collection and authentication of plant and plant materials
- Clean and perform size reduction of plant materials
- Plant material dried in shade and pulverized dried materials
- To perform the extraction and characterisation of ethanolic extract.
- To perform preliminary phytochemical studies
- To evaluate the acute toxicity study of ethanolic extract
- To evaluate in vivo immunomodulatory activity of ethanolic extracts of fruits *Annona reticulata*. Linn by following methods
  - ❖ Delayed type hypersensitivity (DTH) response
  - ❖ Humoral antibody (HA) titer
  - ❖ Total leukocyte count
  - ❖ Determination of total serum protein

## 4. REVIEW OF LITERATURE

Literature review is the first and most important step for the proper selection of plants and it also forms basis for the planning of any scientific work that has to be performed. Due to this reason, the review of literature regarding *Annona reticulata* has been done under various divisions like Pharmacognostical, Phytochemical, Pharmacological, Ethnomedical and also miscellaneous reviews.

### 2.1 Natural Immunomodulators

*Heracleum nepalense* (family Apiaceae) exhibited stimulant property and increased the rate of respiration and blood pressure in goats (Council of Scientific and Industrial Research. The wealth of India: A dictionary of Indian raw material and industrial products. New Delhi: Publication and Information Directorate, Council of Scientific and Industrial Research). An investigation was undertaken to evaluate the immunostimulatory potential of *Heracleum nepalense* roots by using in vitro and in vivo models. The immunostimulatory potential of this plant was investigated by in vitro phagocytic index and lymphocyte viability tests, using IFN- $\alpha$ -2b, a known immunostimulant drug, as the standard. Other tests such as carbon clearance, antibody titer and DTH were studied in mice by using levamisole as the standard. The dried root extract (1000  $\mu$ g/mL) and isolated quercetin glycoside (50  $\mu$ g/mL) significantly increased the in vitro phagocytic index and lymphocyte viability in all assays. They also showed a significant increase in antibody titer, carbon clearance and DTH in mice. Thus *Heracleum nepalense* exhibited a dose-dependent immunostimulant effect, which could be attributed to the flavonoid content or due to the combination with other components (Dash *et al.*, 2006).

*Actinidia macrosperma* (AM) is a medicinal plant in China and has been well known for its activities against cancers, especially of lung, liver and digestive system. The immunomodulatory effects of AM aqueous extract were examined using S180-bearing mice. The immunomodulatory effect was dosedependent in a nonlinear fashion with the optimal dose of 100 mg/kg. The AMinduced antitumor effects were at least partially indirect and were associated with the modulation of immune functions (Lu Y *et al.*, 2007).

*Plumbago zeylanica*, commonly known as Doctorbush, is a species of plumbago with a pantropical distribution (Family: Plumbaginaceae). Effects of seselin identified from *Plumbago zeylanica* on phytohemagglutinin (PHA) stimulated cell proliferation were studied in human peripheral blood mononuclear cells (PBMC). The data demonstrated that seselin inhibited PBMC proliferation-activated with PHA with an IC (50) of 53.87 $\pm$ 0.74 microM. Cell viability test indicated that inhibitory effects of seselin on PBMC proliferation were not through direct cytotoxicity. The action mechanisms of seselin may involve the regulation of cell cycle progression, interleukin-2 (IL-2) and interferon-gamma (IFN-gamma) production in PBMC. Since cell cycle analysis indicated that seselin arrested the cell cycle progression of activated PBMC from the G(1) transition to the S phase. Seselin suppressed IL-2 and IFN-gamma production in a concentration-dependent manner. Furthermore, seselin significantly decreased the IL-2 and IFN-gamma gene expression in PHA-activated PBMC. Therefore, results elucidated for the first time that seselin is likely an immunomodulatory agent for PBMC (Tsai *et al.*, 2008).

*Withania coagulans* (Family Solanaceae) contain six new withanolides, withacoagulins A-F (1-6, resp.), together with ten known withanolides, 7-16, were

isolated from the aerial parts of *Withania coagulans*. These compounds, including the crude extracts of this herb, exhibited strong inhibitory activities on the T- and B-cell proliferation. From, that the results showed *Withania coagulans* has a immunosuppressive activity (**Huang *et al.*, 2009**).

The immunomodulatory effect of clove, *Syzygiurn arornaticurn* (Family: Myrtaceae) essential oils was evaluated by studying humor- and cell-mediated immune responses. Essential oils were administered to mice (once a day, orally, for a week) previously immunized with sheep red blood cells (SRBG5). Glove essential oil increased the total white blood cell (WBG) count and enhanced the delayed-type hypersensitivity (DTH) response in mice. Moreover, it restored cellular and humoral immune responses in cyclophosphamide immunosuppressed mice in a dose-dependent manner. The findings were established that the immunostimulatory activity found in mice treated with clove essential oil is due to improvement in humor- and cell-mediated immune response mechanisms (**Carrasco *et al.*, 2009**).

*Aegle marmelos*, belongs to family Rutaceae is generally known as bael fruit. They were evaluated for potential immunomodulatory activity using the in vitro Polymorphonuclear leukocyte function test. Both Methanolic and Ethanolic extract of *Aegle marmelos* leaves were evaluated for their immunomodulatory activity. The extract was tested for hypersensitivity and hemagglutination reactions; using sheep red blood cells (SRBC) antigen methanol extract exhibited a significant increase in the percentage phagocytosis versus the control. The Methanolic extract was found to stimulate cell mediated and antibody mediated immune responses in rats (**Choubey A *et al.*, 2010**).

To study the immunomodulatory activity of saline extracts of leaves of *Aloe vera* Linn. (Family: Liliaceae) on the albino mice. The saline extract of leaves of *Aloe vera* was administered orally according to their body weight in mice. The assessment of immunomodulatory activity on specific and nonspecific immunity was studied by administration of test extract. The study demonstrates that *A. vera* triggers both specific and non-specific responses to a greater extent. The study comprised the acute toxicity and preliminary phytochemical screening of *A. vera*. From the results obtained and phytochemical studies the immunostimulant effect of *Aloe vera* could be attributed to the alkaloids content (**Atul et al., 2011**).

The immunomodulatory activity of an Indian medicinal plant i.e. ethanolic extract from *Dodonaea viscosa* L.F. namely DV was studied for there phagocytic activity, cell mediated and humoral immune system on rat/mouse. Immunomodulatory effect was assessed in carbon clearance test, delayed type of hypersensitivity (DTH), T-cell population test, and sheep erythrocyte agglutination test (SEAT) in animal treated with DV at doses of 200 and 400 mg/kg. In carbon clearance test, *D. viscosa* exhibited significantly high phagocytic index against control group, indicating stimulation of the reticuloendothelial system. Significant decrease in mean difference, in the foot paw thickness in DTH indicates its anti-inflammatory activity. In SEAT *D. viscosa* treated groups at 200, 400 mg/kg doses showed significant increase in antibody titer against control in normal immune status animals while In T-cell population test, showed significant increase in T-cell rosette formation against control. These results confirm the immunomodulatory activity of *D. viscosa* extract, which is a known immunomodulator in indigenous medicine (**Jagdap et al., 2011**).

## 2.2 *Annona reticulata*

Rasika Dnyandeo Bhalke, Machindra Jayram Chavan, to evaluate possible effects of various extracts of *Annona reticulata* bark on CNS. Petroleum ether, ethyl acetate and methanol extracts of the bark of *Annona reticulata* L. (Annonaceae) were evaluated for analgesic and CNS depressant activities in different animal models. All the extracts exhibited significant central analgesic activity in the hot plate method in mice. All the extract showed statistically significant mild to moderate central nervous system depressant activity assessed by locomotors activity assay and pentobarbitone sleeping time test (**Mahindra Jayram Chavan et al., 2011**)

Jani switu, Harish C R, Mohaddesi Behzad, compared Pharmacognostical study of *Annona squamosa* and *Annona reticulata* leaves belongs to the family Annonaceae. Pharmacognostical study of *Annona squamosa* showed lysogenous cavity and spars trichomes where as *A.reticulata* showed multicellular trichomes filled with tannins and stone cells. The powder charectoristics of *A.squamosa* showed stone cells and prismatic crystals of calcium oxalate where as *A.reticulata* showed pitted stone cells and micro rosettes crystals of calcium oxalate. Annular vessels, lysogenous cavity and paracytic stomata are the common charecters observed in both leaves (**Jani switu et al., 2012**)

Tran Dinh Thang, Ping-Chung Kuo, Guan-Jhong Huang, Nguyen Huy Hung, Bow-Shin Huang, Mei-Lin Yang, Ngo Xuan Luong and Tian-Shung Wu, to the chemical investigation of the leaves of *Annona reticulata* has resulted in the identification of nine compounds, including annonaretin A, a new triterpenoids. The

purified compounds were subjected to the examination of their effects on NO inhibition in LPS-activated mouse peritoneal macrophages and most of them exhibited significant NO inhibition, with IC<sub>50</sub> values in the range of  $48.6 \pm 1.2$  and  $99.8 \pm 0.4$   $\mu$ M (**Tian-Shung Wu *et al.*, 2013**)

Vishal Kumar Soni, Manisha Pathak, Dinesh Kumar Yadav, Rakesh Maurya, Mahendra Sahai, Swatantra Kumar Jain and Shailja Misra-Bhattacharya, reported that twig of *A.recticulata* contain a large number of alkaloids, we chose to study its medicinal properties on the immune response in mice. The present study, thus, aims at evaluation of immunomodulatory activity in the crude ethanolic extract and its four fractions, viz. hexane (F1), chloroform (F2), n-butanol (F3) and aqueous (F4) prepared from the twigs of AS to locate the active constituents in the fractions. The extract and fractions were fed orally at 3, 10 and 30 mg/kg for 14 consecutive days and mice were euthanized to assess various immune parameters. The ethanolic extract and its three fractions F2, F3 and F4 were found active since they increased splenic T and B cellular proliferation with a significant accentuation in peritoneal macrophage function, differentially increased the CD4+, CD8+ T lymphocytes and CD19+ B lymphocytes. The extract and its active fractions also demonstrated significant Th1 or Th2 mixed cytokine response at almost all doses tried in a dose-dependent manner. Its hexane fraction, however, could only induce reactive oxygen species production in peritoneal macrophages and could not induce lymphocytes; thus, it remained inactive. Thus, the activity could be localized distributed in its three fractions (chloroform, n-butanol and aqueous). Further purification and evaluation of the active molecule/s is underway in our laboratory. (**Shailja Misra-Bhattacharya *et al.*, 2013**)



Kalyani Pathak, Kamaruz Zaman, reported that medicinal importance of whole plants of *Annona reticulata* linn is a highly apparent plant in ayurvedic system of medicine for the treatment of various ailments. The plant is traditionally used for the treatment of epilepsy, dysentery, cardiac problems, worm infestation, constipation, haemorrhage, antibacterial infection, dysuria, fever, ulcer etc. It also has antifertility, antitumour and abortifacient properties. This plant is reputed to possess varied medicinal properties. Several research workers investigated the pharmacological activities of different parts of the plant. Present review gives an overview on botanical description, ethnomedical and therapeutic importance and chemical constituents (**Kalyani Pathak *et al.*, 2013**)

C.A. Gonçalves, N.L. Silva, M.O. Mauro, N. David, A.L. Cunha-Laura, S.A. Auharek, A.C.D. Monreal, M.C. Vieira, D.B. Silva, F.J.L. Santos, J.M. Siqueira and R.J. Oliveira, Evaluate mutagenic, teratogenic, and immunomodulatory effects of *Annona nutans* hydromethanolic fraction on pregnant mice. To analyze the potential toxic effects of medicinal plants during gestation. The present study aimed to evaluate the effects of *A. nutans* hydromethanolic fraction leaves (ANHMF) on mutagenic and immunomodulatory activity, reproductive performance, and embryo-fetal development in pregnant female mice. The animals (N = 50 female and 25 male) were divided into 5 groups: Control, Pre-treatment, Organogenesis, Gestational, and Pre+Gestational. The results indicate that ANHMF mainly contains flavonoid and other phenolic derivatives. It was found that it does not exhibit any mutagenic or immunomodulatory activity and it does not cause embryo-fetal toxicity. Based on the protocols used in the present studies, our analyses confirm that it is safe to use ANHMF during pregnancy (**R.J. Oliveira *et al.*, 2014**)

Balu Selvakumar, Gokulakrishnan J, Elanchezhian K and Deepa J, reported that leaves of *A.reticulata* showed mosquito larvicidal, ovicidal and pupicidal activities on *Anopheles stephensi* and *Culex quinquefasciatus*. *A. reticulata* benzene, chloroform, ethyl acetate and methanol extract were tested against 3rd instar larvae of *Ae. aegypti*, *An. stephensi* and *C. quinquefasciatus* for 24 hr and mortality were recorded at various concentrations. The LC50 and LC90 values were determined following probit analysis. The ovicidal activity was determined against *Aedes aegypti*, *Anopheles stephensi* and *Culexquinquefasciatus* exposed to various concentrations were tested under laboratory conditions. The LC50 and LC90 values of benzene, chloroform, ethyl acetate and methanol extract of *A. reticulata* against early third instar larvae of *Ae. Aegypti*, *An. stephensi* and *Cx. Quinquefasciatus*. Methanol extracts showed maximum ovicidal activity followed by benzene, chloroform and ethyl acetate against selected vector mosquitoes. In pupicidal activity, among the four solvent extracts tested against selected mosquitoes at 200ppm higher concentrations, the methanol was found to be most effective for pupicidal activity provided against *C. quinquefasciatus*, *Ae. aegypti* and *An. stephensi* respectively. The present investigation lead the path of exploration of *Annona reticulata* for eradication of selected medically important human vector mosquitoes, thereby, gaining a real momentum to include this plant product for intense vector control programme (BalusSelvakumar *et al.*, 2015)

Lauve Rachel Tchokouaha Yamthe, Patrick Valere Tsouh Fokou, Cedric Derick Jiatsa Mbouna, Fabrice Fekam Boyom, reported that antimalarial activity of ethanolic extract of *Annona muricata* and *Annona recticulata*. Cytotoxicity studies on erythrocytes and Human Foreskin Fibroblasts cells and against the W2 strain of

*P. falciparum* in culture. IC<sub>50</sub> values of extract ranging from 0.07 to 3.46 µg/mL. The most potent was the subfraction 30 from *A. muricata* stem bark (IC<sub>50</sub> = 0.07 µg/mL) with a selectivity index of > 142. Subfraction 3 from *A. muricata* root also exhibited very good activity (IC<sub>50</sub> = 0.09 µg/mL) with a high selectivity index (SI > 111). Amongst the isolated compounds, only gallic acid showed activity with IC<sub>50</sub> of 3.32 µg/mL and SI > 10. These results support traditional claims for *A. muricata* and *A. reticulata* in the treatment of malaria (**Fabrice Fekam Boyom et al., 2015**)

Ruchi. N. desai, pankaj. K. hiradhar, to reported that larvicidal activity of methanolic extract of leaves of *Annona reticulata*, active against a limited number of species including specific target insects, less expensive, easily biodegradable to non-toxic products, and potentially suitable for use in mosquito control programme. The present study clearly suggests the efficacy of *A. reticulata* extract on larvae of *C. quiquefasciatus*. Thus there are all chances of exploring this plant for its pupicidal, antifeedent, adulticidal, mode of action, synergism with the biocides under field condition and various other aspects which can be commercially used instead of the synthetic chemicals found in the market which are tremendously harmful to the whole ecosystem (**Ruchi N Desai et al., 2015**)

## 5. PLANT PROFILE



**Fig. 4 Whole Plant**



**Fig. 5 Fruits**

### Scientific Classification

Domain	: Eukaryota
Kingdom	: Plantae
Class	: Angiosperms
Division	: Magnolids
Order	: Magnoliales
Family	: Annonaceae
Genus	: Annona
Species	: Reticulata
Botanical name	: Annona reticulata
Common names	: Custard apple

### Synonyms

Shubha, Sitaphala, *Annona excelsa* Kunt, *Annona laevis* Kunth, *Annona longifolia* Moc, *Annona longifolia* Sesse, *Annona riparia* kunth.

**Vernacular Names**

English	: Custard apple, wild sweet sop
Hindi	: Ramaphal
Telugu	: Ramaphalamu
Assamese	: Atlas, Ata
Gujrat	: Sitaphal
Punjab	: Sharifa
Oriya	: Ato

**5.1 Botanical Description**

A tree about 6m high. Bark thin and grey. Leaves simple, alternate, 3.5-8 x1.5-4 cm, oblong – lanceolate or elliptic, obtuse or subacute, pellucidpunctate, glabrous above, glaucous and pubescent beneath when young; lateral nerves 8-11 pairs, petiole upto 2 cm long. Flower bisexual, drooping, green, solitary, leaf opposed or 2-4 on short extra axillary branchlets. Fruit globose, 5-10 cm in diameter, usually with a glucose bloom on the surface when young, yellowish-green when ripe, easily broken into large pieces; areoles well marked, pulp white, sweet. Many seeds, arilate, brownish-black, smooth or polished and hard. (**Kamaruz Zaman *et al.*, 2013**).

**Flowering:** March – July

**Fruiting:** August –January

## 5.2 Distribution

It is found wildly and cultivated throughout India upto an altitude of 900m. It is found growing gregariously and widely in the hilly tracts, waste lands and has become completely naturalized in several districts of Andhra Pradesh, Punjab, Rajasthan, Uttar Pradesh, Madhya Pradesh, Bihar, West Bengal, Assam, Gujarat, Maharashtra, Karnataka, Kerala and Tamil Nadu. It is a native of South America and West Indies.

## 5.3 Medicinal Uses

*Annona reticulata* is a highly apparent plant in ayurvedic system of medicine for the treatment of various ailments. The plant is traditionally used for the treatment of epilepsy, dysentery, cardiac problems, worm infestation, constipation, haemorrhage, antibacterial infection, dysuria, fever, and ulcer. It also has antifertility, antitumour and abortifacient properties (**Surendra *et al.*, 2013**).

## 6. MATERIALS AND METHODS

### Collection of Materials, Chemicals and Drugs

- The Sheep Red Blood Cells (SRBCs) were procured from Veterinary College Hebbal (KVAFSU), Bangalore.
- The Levamisole (Cipla Limited- India) was purchased from local pharmacy, Bangalore.
- The Humoral antibody tests were performed in SAROJ diagnostic laboratories. All chemicals were procured from SAROJ diagnostic laboratories, Calicut.

### Collection and Authentication of *Annona Reticulata* Fruits

- Date of collection: 23- 01- 2016.
- Place of collection: Feroke, Kozhikode dist, Kerala.
- Time of collection: Early morning hours (6 - 8 am).

Fruits of *Annona reticulata* were collected from Feroke Kozhikode. The plant material was taxonomically identified by botanist Mr. A. K Pradeep kumar, Assistant Professor, Department of Botany, University of Calicut The drug material was dried under shade for about 14 days, powdered and stored in an air tight container.

**PHYSICOCHEMICAL EVALUATION OF THE CRUDE DRUG (IP 2006)****a) Evaluation of foreign matter**

A quantity of 50g of fruits to be examined was weighed and it was spread out in a thin layer. The foreign matter was detected by inspection with the naked eye. It was separated and weighed and percentage of foreign matter was calculated.

**b) Moisture content (loss on drying)**

fresh fruits of *Annona reticulata* was weighed and dried in a hot air oven at 100°C until a constant weight was obtained. The loss in weight was recorded as moisture content.

**ASH VALUE (Kokate *et al*, 1985)****Principle**

The ash content of a crude drug is generally taken to be the residue remaining after incineration. It usually represents the inorganic salts naturally occurring in the drug and adhering to it, but it may also include inorganic matter added for the purpose of adulteration. There is a considerable difference varies within narrow limits in the case of the same individual drug. Hence an ash determination furnishes a basis for judging the identity and cleanliness of a drug and gives information relative to its adulteration with inorganic matter. Ash standards have been established for a number of official drugs. Usually these standards get a maximum limit on the total ash or on the acid insoluble ash permitted.

The total ash is the residue remaining after incineration. The acid insoluble ash is the part of the total ash which is insoluble in diluted hydrochloric acid.



The ash or residue yielded by an organic chemical compound is as a rule, a measure of the amount of inorganic matters present as impurity. In most cases, the inorganic matter is present in small amounts which are difficult to remove in the purification process and which are not objectionable if only traces are present. Ash values are helpful in determining the quality and purity of the crude drugs in powder form.

Procedures given in Indian pharmacopoeia were used to determine the different ash values such as total ash and acid insoluble ash.

### **Total Ash**

Weighed accurately about 3 gm of dried powdered drug was taken in a tarred silica crucible and incinerated by gradually increasing the temperature to make it dull red until free from carbon cooled and weighted and then calculated the percentage of total ash with reference to the air dried drug.

### **Acid Insoluble Ash**

The ash obtained as directed under total ash above was boiled with 25 ml of 2N HCl for 5 minutes. The insoluble matter was collected on ash less filter paper, washed with hot water ignited and weighed, then calculated the percentage of acid insoluble ash with reference to the air dried drug.

### **Water Soluble Ash**

The total ash obtained was boiled with 25 ml of water for 5 minutes. The insoluble matter was collected on an ash less filter paper, washed with hot water and ignited for 15 minutes at a temperature not exceeding 450°C. The weight of

insoluble matter was subtracted from the weight of total ash. The difference in weight represents the water soluble ash. The percentage of water soluble ash calculated with reference to the air dried drug.

### **Extractive Values**

Extractive values of crude drugs are useful for their evaluation, especially when the constituents of a drug cannot be readily estimated by any other means. Further, these values indicate the nature of the constituents present in a crude drug.

### **Determination of Alcohol Soluble Extractive Value**

5 gm of the air-dried coarse powder of *Annona reticulata* was macerated with 100 ml of 90% ethanol in a closed flask for 24 hours, shaking frequently during the first 6 hours and allowing standing for 18 hours. Thereafter, it was filtered rapidly taking precautions against the loss of the solvent. Out of that filtrate, 25 ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105°C and weighed. The percentage of ethanol soluble extractive value was calculated with reference to the air- dried drug.

### **Determination of Water Soluble Extractive Value**

Weigh accurately 5 gm of coarsely powdered drug and macerate it with 100 ml of water in a closed flask for 24 hours, shaking frequently during the first 6 hours and allow to standing for 18 hours. Thereafter, it was filtered rapidly. Then 25 ml of the filtrate was evaporated to dryness in a tarred flat bottomed shallow dish, dried at 105°C and weighed. The percentage of water soluble extractive was calculated with reference to the air dried drug.

## 6.1 Extraction

Extraction is the separation of medicinally active portions of plant tissues using selective solvents through standard procedures. Such extraction techniques separate the soluble plant metabolites and leave behind the insoluble cellular marc. The products so obtained from plants are relatively complex mixtures of metabolites, in liquid or semisolid state or (after removing the solvent) in dry powder form. The general techniques of medicinal plant extraction include maceration, infusion, percolation, digestion, decoction, hot continuous extraction (Soxhlet), aqueous-alcoholic extraction by fermentation, counter-current extraction, microwave assisted extraction, ultrasound extraction (sonication), supercritical fluid extraction, and phytonic extraction (with hydrofluorocarbon solvents) (**Prashant *et al.*, 2011**).

**Soxhlet Extraction** In this method, the finely ground crude drug is placed in a porous bag or “thimble” made of strong filter paper, which is placed in the middle chamber of the Soxhlet apparatus. The solvent taken in the round bottom flask is heated, and its vapours get condensed in the condenser. The condensed solvent drips into the thimble containing the crude drug, and extracts it by contact. When the level of liquid in middle chamber rises to the top of siphon tube, the liquid contents of chamber siphon back into round bottom flask. This process is continuous and is carried out till extraction is complete. The advantage of this method, compared to previously described methods, is that large amounts of drug can be extracted with a much smaller quantity of solvent. This affects tremendous economy in terms of time, energy and consequently financial inputs. At small scale, it is employed as a batch process only, but it becomes much more economical and viable when

converted into a continuous extraction procedure on medium or large scale (**Handa, 2008**).

## 6.2 PHYTOCHEMICAL SCREENING

Phytochemical screening includes the following steps

- Preliminary Phytochemical screening
- Chromatographic and spectroscopic studies.
- Estimation of total Flavanoids and Phenolic contents.

### Preliminary Phytochemical screening

Qualitative analysis for determining the presence of alkaloids, tannins, Flavanoids, terpenoids , steroids, glycosides, saponins, resin, and oil in the plant extracts, were carried out using standard methods as described by **Harborne (1973)**, **Trease and Evans (1978)** and **Sofowora (1993)**. 0.5 gm of the dried extracts were dissolved in 20 ml distilled water, filtered and used for various qualitative tests. The following chemical testes were carried out using extracts of *Curcuma aeruginosa* rhizome extracts. (**Khandelwal, 1998; Kokate, 1993**).

### *Chemical test for alkaloids*

The extracts were evaporated and to the residue dilute hydrochloric acid was added and filtered. With the filtrate, the following tests were performed

#### a) Mayer's Test

To 2-3 ml of extract, added few drops of Mayer's reagent. The formation of white or creamy precipitate indicates the presence of alkaloids.

**b) Wagner's Test**

To 2-3 ml of the extract, added few drops of Wagner's reagent. Formation of reddish brown precipitate indicates the presence of alkaloids.

**c) Hager's Test**

To 2-3 ml of extract, added few drops of Hager's reagent. A prominent yellow precipitate indicates the presence of alkaloids.

**d) Dragendorff's Test**

To 2-3 ml of extract, added few drops of Dragendorff's reagent. A prominent orange brown precipitate confirms the presence of alkaloids.

***Chemical tests for glycosides*****a) Legal's Test**

To the extract, added 1 ml of pyridine and 1 ml of sodium nitroprusside. A pink to red colour indicates the presence of glycosides.

**b) Baljet's Test**

To a few ml of the extract, add 1 ml sodium picrate solution. Yellow to orange colour reveals the presence of cardiac glycosides.

***Chemical tests for phenolics*****a) Ferric chloride test**

To 2-3 ml of the extract, add 5% ferric chloride, formation of deep blue-black colour indicates the presence of phenolic compounds.

**b) Lead acetate test**

To small quantity of extract, added 3 ml of 10% lead acetate solution was added to it. A bulky white precipitate indicates the presence of phenolic compounds.

***Chemical tests for flavonoids*****a) Aqueous sodium hydroxide test**

To the residue, addition of increasing amount of sodium hydroxide shows yellow colouration, which decolourises after addition of acid indicates the presence of flavonoids.

**b) Shinoda test**

To the extract, added 5 ml of 95% ethanol, few drops of concentrated hydrochloric acid and 0.5 g of magnesium turnings. The presence of pink colour indicates the presence of flavonoids.

***Chemical tests for carbohydrates*****a) Molisch's Test**

To 2-3 ml of extract added few drops of alpha-naphthol solution in alcohol, shaken and added conc. sulphuric acid from the sides of the test tube. A violet ring is formed at the junction of two liquids indicates the presence of carbohydrates.

**b) Fehling's Test**

1 ml of Fehling's A and 1 ml of Fehling's B solutions was mixed and boiled for one minute. Added equal volume of test solution and heated in boiling water bath for 5-10 min. First a yellow, then brick red precipitate is observed, indicates the presence of reducing sugars.

**c) Benedict's Test**

Equal volume of Benedict's reagent and test solution were mixed, heated in boiling water bath for 5 min. Solution appears green, yellow and red depending on amount of reducing sugar present in test solution.

***Chemical tests for proteins and amino acids*****a) Millon's Test**

3 ml of test solution was mixed with 5 ml millon's reagent. White precipitate form and upon warming precipitate turns brick red or the precipitate dissolves giving red coloured solution indicates the presence of proteins.

**b) Biuret Test**

3 ml of the test solution was added to 4% sodium hydroxide and few drops of 1% copper sulphate solution. Appearance of violet or pink colour indicates the presence of proteins.

**c) Ninhydrin Test**

3 ml of the test solution was heated with 3 drops of 5% Ninhydrin solution in boiling water bath for 10 min. Appearance of purple or bluish colour indicates the presence of amino acids.

***Chemical test for terpenoids***

3 ml of the extract was added to 2 ml of chloroform. Then 3 ml of concentrated sulphuric acid was added carefully to form a layer. A reddish brown coloration at the interface indicates the presence of terpenoids.

***Chemical Tests for Steroids*****a) Libermann - Burchard's Test**

2 ml of the extract was mixed with chloroform, added 1-2 ml of acetic anhydride and 2 drops of conc. Sulphuric acid from the sides of the test tube. Appearance of first red, then blue and finally green colour indicates the presence of steroids.

**b) Salkowski Test**

2 ml of the extract was mixed with 2 ml chloroform and 2 ml concentrated sulphuric acid, shaken. The appearance of red colour on chloroform and greenish yellow fluorescence on acid layers indicate the presence of steroids.



### ***Chemical Tests For Saponins***

#### **a) Foam or Froth Test**

1 ml of the extracts were diluted with water to 20 ml and shaken in a graduated cylinder for 15 minutes. One centimetre layer of foam indicates the presence of saponins.

#### **Chemical Test for Sterols**

- **Libermann-Burchard test:** Mix 2ml of extract with few drops of chloroform and acetic anhydride and 2 drop sulphuric acid along the side of test tube first blue then red finally green colour appears.
- **Salkowski's test:** 2ml of extract were treated in 2ml chloroform and 2ml of concentrated sulphuric acid, shaken well and red colour appeared in chloroform layer and greenish yellow fluorescence in acidic layer.
- **Chemical Test for Terpenoids:** Extract added with chloroform then adds 4ml of conc: sulphuric acid carefully to form a layer. Reddish brown coloration at the interface shows the presence of terpenoids.

### **6.3 Acute Toxicity Study**

Acute toxicity for ethanolic extract of fruits will be done according to the office of pollution prevention and toxics (OPPT) The overnight fasted rat is weighed and selected. The extracts will be dosed in a stepwise procedure, by using up and down or stair case method. The two animals selected with a dose of 50 mg/kg. Orally and examined for 24h for mortality. Subsequent dose are then increased to attain maximum non lethal and minimum lethal dose. The extract was found to be

safe at the dose of 5g/kg per oral. Maximum safe dose (5g/kg) corresponding to 500 mg/kg and 100 mg/kg were selected as high and low doses respectively.

### **Experimental Animals**

The experiment was carried out by using Wistar rats, which were procured from central animal house of the Institute. The experimental protocol has been approved by institutional animal ethical committee, Komarapalayam. Rats of Wistar strain weighing between 150 to 200 gm were maintained under standard laboratory conditions. They were provided with a standard diet supplied by Pranav agro industries Ltd India.

### **Experimental Protocol**

24 rats were divided into four groups

Group I : Control (normal saline)

Group II : *Annona reticulata* ethanolic extract was administered at a dose 100 mg/kg/day by oral route

Group III : *Annona reticulata* ethanolic extract was administered at a dose 500 mg/kg/day by oral route

Group IV : Standard- Levamisole was administered at a dose of 50mg/kg/day by oral route for 14 days.

## 6.4 Experimental Setup

The animal model is required to study the following methods

- Delayed type hypersensitivity (DTH) response
- Humoral antibody (HA) titer
- Total leukocyte count
- Determination of total serum protein and albumin -globulin ratio

### 6.4.1 Delayed Type Hypersensitivity (DTH) Response

For the evaluation of delayed type of hypersensitivity (DTH) test animals were divided in to four groups, having six animals in each. Group I, the control, was given 2ml of 5% normal saline and to group II,III, IV was administered of 100 mg and 500 mg/kg body weight of ethanolic extract intraperitonially for ten days. On 10th day 0.1ml of SRBC solution was injected subcutaneously in to the right footpad. After 24,48,72,96 hrs, thickness of footpad was measured by plethisometer. Difference in the footpad thickness in control and treated group has been taken as the measure of the DTH reaction (**Dikshit *et al.*, 2000**)

### 6.4.2 Humoral Antibody Titre

The animals were immunized by injecting 0.1 ml of SRBCs suspension, containing  $1 \times 10^8$  cells, intraperitonially, on day 0. Blood samples were collected in micro centrifuge tubes from individual animals of all the groups by retro orbital vein puncture on day 10. The blood samples were centrifuged and the serum separated. Antibody levels were determined by the hemagglutination technique.

**Method for Serial dilution**

This was performed by using 96 wells (12x8) U bottomed titre plate. The wells were marked from I to XII. In the first (I) and last well (XII) 25 microliter of serum collected from treated animals was added and inactivated at 56 degree Celsius for 30 minutes. Afterwards to all the wells except well number XII, 25 microliter of PBS was added. 25 microliter was taken from first well and added to 2<sup>nd</sup> well again 25 microliter from second well was taken and added to third well and continued the same procedure up to well number XI. After this 25 microliter of sample from well number XI was discarded. Finally 25 microliter of 1% SRBC was added to all the wells and was kept at room temperature for two hours (**Vinod S Pawar *et al.*, 2012**)

**6.4.3 Total Leukocyte Count**

W.B.C diluting pipette: It has got three graduations. Two graduations 0.5 and 1 are present on the stem of the pipette and the third mark 11 is placed just above the bulb. Blood is drawn up to mark 0.5 and the rest of the bulb is filled by sucking up diluting solution up to the mark 11, the bulb of the pipette is so constructed that it holds exactly 20 times the volume of fluid contained in the stem of the pipette up to mark 1. Although fluid is drawn up to 11, the dilution of the blood will be 20 because the last part of the fluid remains locked up in the stem and is not available for dilution

**The Counting Chamber**

The ruling area consists of 9 square millimeters. The central of the smallest squares are separated by triple lines in which RBC will be counted. The side of each square for counting WBC is  $\frac{1}{4}$  mm.

**Diluting Fluid for WBC (Turks fluid)**

Commonly the fluid is made up as follows

Glacial acetic acid : 1.5ml

1% solution of gentian violet in water : 1ml

Distilled water : 98ml

The glacial acetic acid haemolysis the red cells, while the gentian violet stains the nucleus of leukocytes

**Method of Counting W.B.C**

The white cells are counted in four corners of 1 square millimeter ruled area on both sides. The white cells are recognized by the retractile appearance and by the slight color given to them by the stain contained in the diluting fluid. The cells touching the left side and upper side of boundary line are not counted

**Calculations**

The area of the smallest =  $1/16 \text{ mm}^2$

Volume of smallest square =  $1/160 \text{ mm}^3$

Total number of squares counted =  $16 \times 4 = 64$

Total number of cells counted = X

$64/160 \text{ mm}^3$  of diluted blood contains = X cells

So,  $1 \text{ mm}^3$  of diluted blood contains =  $160/64 \times X$  cells

$1 \text{ mm}^3$  of undiluted blood contains =  $160/64 \times 20 \times X$  cells.

**6.4.4 Determination of Total Serum Protein****Total Protein (Biuret Method)**

Total Protein-To exactly 4 cc. of 10 per cent sodium hydroxide in a 10 ml standard flask and add 0.1 cc. of fresh serum with a Folin micropipette. Rinse out the pipette three times with sodium hydroxide solution. Mix by rotating and add 0.5 cc. of 1 per cent copper sulphate. Shake vigorously five to six times. Allow to stand for 25 minutes and absorbance read in a U.V Spectrophotometer at 540 nm.

## 7. RESULTS

### 7.1 PHYSICOCHEMICAL EVALUATION OF THE DRUG

#### Evaluation of foreign matter

Foreign matter of fresh fruits of *Annona reticulata* was found to be 9.82.

#### Moisture content (Loss on drying)

Moisture content of fresh fruits of *Annona reticulata* was found to be 53.1

#### Extractive value

Water soluble extract - 7.83%

Alcohol soluble extract - 6.37%

#### Ash value of the drug

Total ash - 5.8%

Acid insoluble ash - 0.61%

Water soluble ash - 3.71%

### 7.2 Phytochemical Screening

In preliminary phytochemical analysis was done on the basis of standard procedure. Show presence of secondary metabolites. They were shown in table no.1

**Table. 1.** Preliminary phytochemical screening of ethanolic extract of *Annona reticulata* fruits.

Sl.no	Phytoconstituents	Ethanolic extract
1	Alkaloids	+
2	Glycosides	-
3	Flavanoids	+
4	Phenols	+
5	Saponins	+
6	Steroid	-
7	Tannins	-
8	Terpenoids	+
9	Carbohydrates	+

## 7.2 Acute toxicity study

For toxicity studies, crude ethanolic extract were administered orally to the three groups having two rats in each with graded doses (50mg/kg-500mg/kg body weight) of *Annona reticulata*. Mortality rates were observed after 7 days (Choudhary *et al.*, 1997)



**Table: 2 Determination of acute toxicity of ethanolic extract of *Annona reticulata***

Sl. No	Dose (mg/kg. Body weight)	Percent mortality
1	50	0
2	100	0
3	150	0
4	200	0
5	250	0
6	300	0
7	350	0
8	400	0
9	450	0
10	500	0

### 7.3 Determination of Delayed Type Hypersensitivity Response

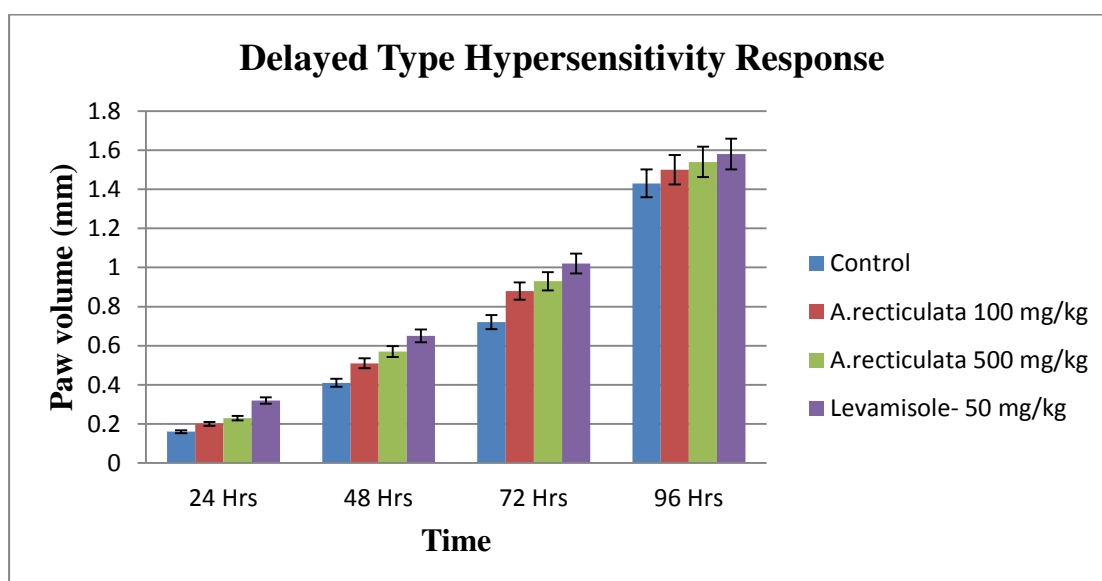
The effect of test extract and standard drugs on the DTH response in wistar rats using SRBCs as antigen, administration of ethanolic extract of *Annona reticulata* at the dose of 100mg/Kg and 500mg/Kg and Levamisole 50mg/Kg treatments which were given orally. After 24, 48, 72, 96 hrs showed significant increase in paw edema compared to control group.

**Table.3 Effect of crude ethanolic extract of *Annona reticulata* on delayed type of hypersensitivity response**

Sl. No	Groups	Paw volume (mm)			
		24 Hrs	48 Hrs	72 Hrs	96 Hrs
I	Control	1.43±0.027	0.72±0.019	0.41±0.013	0.16±0.012
II	Crude ethanolic extract (100 mg/kg body weight )	1.50±0.011	0.88±0.018	0.51±0.015	0.20±0.021*
III	Crude ethanolic extract (500 mg/kg body weight )	1.54±0.016	0.93±0.019	0.57±0.012	0.23±0.014**
IV	Standard – Levamisole (50 mg/kg body weight)	1.58±0.010	1.02±0.029	0.65±0.021	0.32±0.016***

n= 6. Tabulation values represents mean ± SD (\*P<0.05, \*\*P<0.025, \*\*\*P<0.001)

**Fig. 6 Show Delayed Type Hypersensitivity response**



**Fig. 7 Paw edema observed in animals after injecting sheep's RBC****Control animal*****Annona reticulata* 100 mg/kg*****Annona reticulata* 500 mg/kg.****Levamisole 50 mg/kg**

#### **7.4 Humoral Antibody Titre**

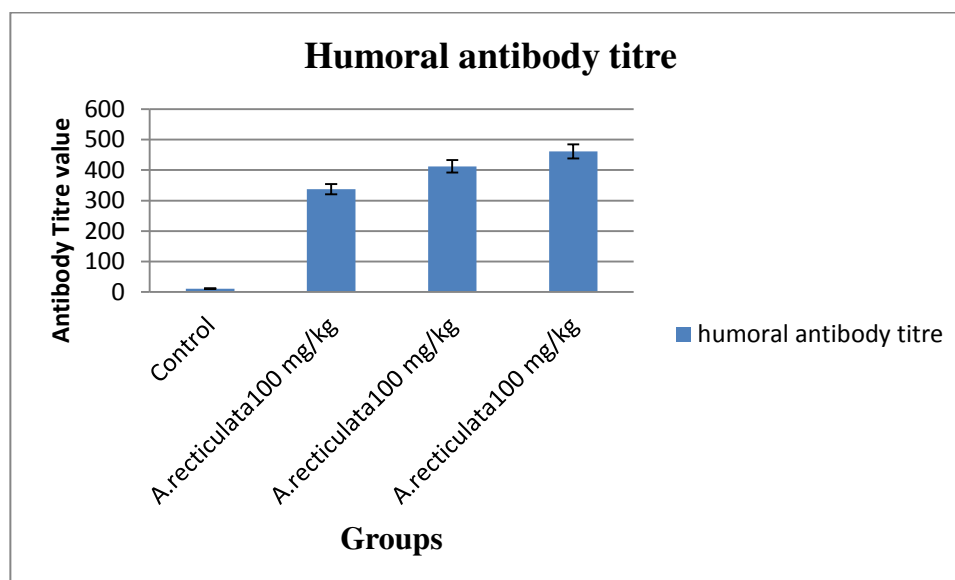
Administration of ethanolic extract of at the dose of (100 & 500 mg/kg) and Levamisole 50mg/Kg treatments which were given orally for 14 days showed highly significant increase in antibody titre values compared to control group. The results are shown in below table 4

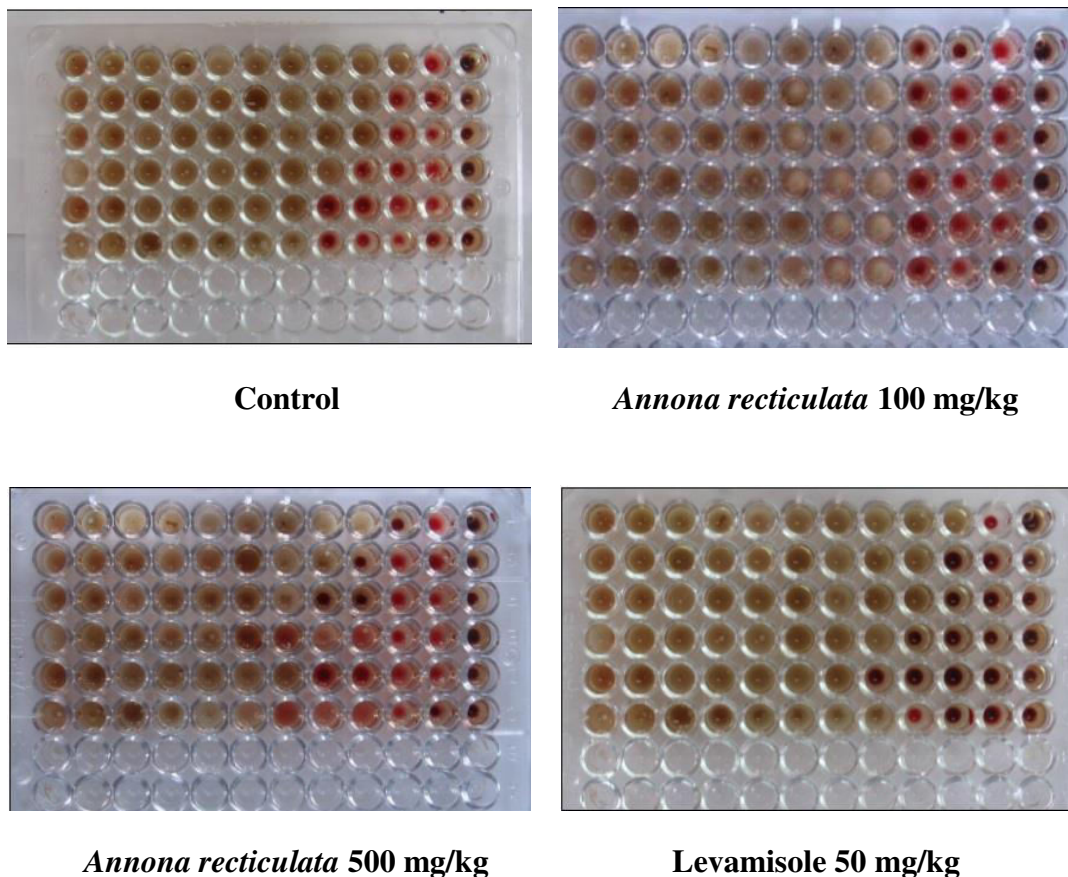
**Table.4 Effect of crude ethanolic extract of *Annona reticulata* of Humoral Antibody titre**

Sl. No	Group	Humoral antibody titre
I	Control	11±1.0210
II	<i>Annona reticulata</i> 100 mg/kg body weight	337.47±2.0401*
III	<i>Annona reticulata</i> 500 mg/kg body weight	412±1.5010**
IV	Levamisole 50 mg/kg	461± 2.6861***

n=6, humoral antibody titre value mean ± SEM (\*P<0.05, \*\*P<0.01, \*\*\*P<0.001)

**Fig.8 Show Humoral Antibody Titre**



**Fig. 9 Button formation of Antibody titre on Microtitre plate**

### 7.5 Total Leukocyte Count

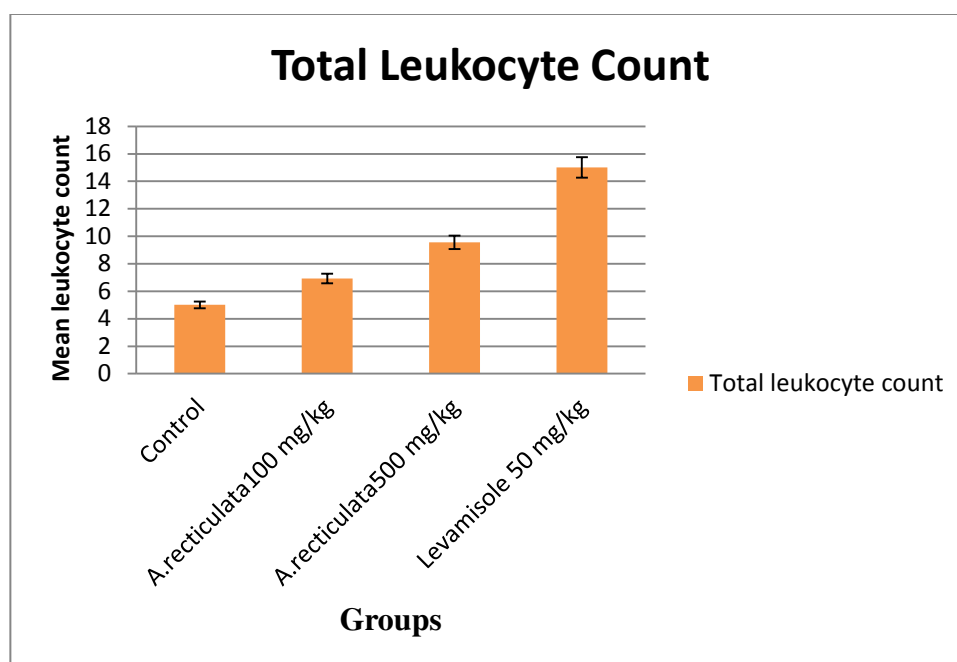
The effect of test extract and standard drugs on Total Leukocytes in wistar rats, administration of ethanolic extract of *Annona recticulata* at the dose of (100,500 mg/kg) and Levamisole 50mg/Kg treatments which were given orally for 14 days. The low dose of extract (100 mg/kg) show effect on TLC count compared to control group, whereas the 500mg/Kg and standard drug Levamisole 50mg/Kg showed significant increase in total leukocytes count values compared to control group. The results are shown in below table 5.

**Table .5 Effect of crude ethanolic extract of *Annona reticulata* of Humoral Antibody titre.**

Sl. No	Group	Mean Leukocyte count
I	Control	$5.01 \times 10^3$ cu.mm $\pm$ 0.2640
II	<i>Annona reticulata</i> 100 mg/kg	$6.93 \times 10^3$ cu.mm $\pm$ 0.2461*
III	<i>Annona reticulata</i> 500 mg/kg	$9.56 \times 10^3$ cu.mm $\pm$ 0.3101**
IV	Levamisole 50 mg/kg	$15.01 \times 10^3$ cu.mm $\pm$ 0.1381***

n= 6, total leukocyte count means  $\pm$ SEM (\*P<0.05, \*\*P<0.01, \*\*\*P<0.001)

**Fig. 10 Show Total Leukocyte Count**



### 7.6 Determination of Total Serum Protein

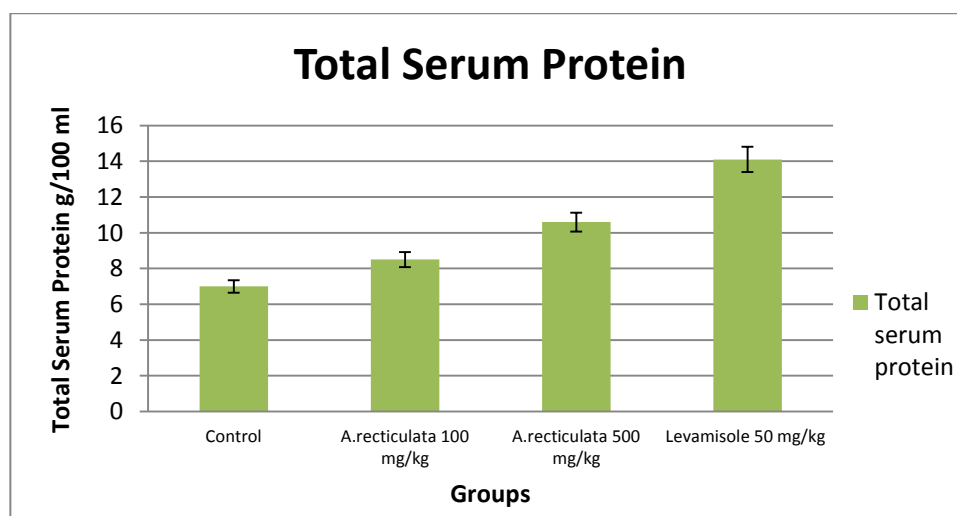
The effect of test extract and standard drugs on Total serum protein in wistar rats, administration of ethanolic extract of *Annona reticulata* at the dose of (100,500 mg/kg) and Levamisole 50mg/Kg treatments which were given orally for 14 days. The low dose of extract (100 mg/kg) and large dose (500 mg/kg) standard drug Levamisole 50mg/Kg showed significant increase in total serum values compared to control group. The results are shown in below table 5.

**Table 6. Effect of crude ethanolic extract of *Annona reticulata* of total serum protein**

Sl. No	Group	Total serum protein (g/100 ml)
I	Control	7±0.1201
II	<i>Annona reticulata</i> 100 mg/kg	8.5±0.952
III	<i>Annona reticulata</i> 500 mg/kg	10.6±0.1012
IV	Levamisole 50 mg/kg	14.1±0.010

n= 6, total serum value means  $\pm$  S,D

**Fig. 11 Show Total Serum Protein**



## 7. DISCUSSION

Plants serve as vast source for varied phytoconstituents exhibiting varied pharmacological property. Identifying such potential plants is of significance in medicine. In the treatment of a disease the toxicity and resistance of available drugs are the major world-wide problem. Because of these the designs of new drug which can overcome resistance as well as toxicity become one of the leading areas of drug design.

In light of this, in the present study; widely available herb *Annona reticulata* was selected for Pharmacognostical standardization according to WHO guidelines, thorough phytochemical analysis followed by pharmacological screening for immunomodulatory activity in Wistar rats.

After the standardization, the shade dried plant material was subjected to detailed phytochemical analysis. The various chemical and acid treatments of the fruits of the study established the identity of categories of phytoconstituents. The fruits were subjected to soxhlet extraction using ethanol as solvent. Later the extracts were subjected to preliminary phytochemical analysis to ascertain the presence of various categories of phytoconstituents. The preliminary phytochemical screening revealed the significant presence of alkaloids, trepenoids, saponnins, flavanoids, tannins, phenolic compounds and carbohydrates.

Acute toxicity study was evaluated by using ethanolic extract on Wistar rats, and increasing the concentration of plant extract found to be most effective at low dose (100 mg/kg), where as high dose (500 mg/kg) of ethanolic extract of *Annona reticulata* was moderately effective in modulating immune system.



The study was carried out using four different methods, each of which provides information about effect on different components of the immune system (Wagner 1984)

Delayed Type Hypersensitivity Test was done to study the effect at crude aqueous and crude ethanolic extract on cell-mediated immune response to paw edema in 24, 48 hrs and then after 72 and 96 hrs paw volume significantly increase when compared with control. But in case of standard drug (Levamisole) compared with test drug, standard drug show significance paw volume. ethanolic extract of at the dose of (100 & 500 mg/kg) and Levamisole 50mg/Kg treatments which were given orally for 14 days showed highly significant increase in antibody titre values compared to control group

Humoral antibody titer assay is one of the key parameter used to assess the humoral immune response of the animal. As the antigen is expected to induce the production of antiserum against it, in the present study sheep red blood cells were used to elucidate the production of antibody against RBC. In a individual where immune system is primed antibody against a particular antigen is expected to be at higher titer. Accordingly in the present study a very high humoral antibody titer was recorded and group II and group III individual which received the lowest concentration (100 mg/kg and 500 mg/kg) of test drug. On the contrary higher concentrations of the drug have surprisingly reduced the HA titer. **Pradhan et al (2009)** administered extract of herbal product to albino rats showed a increased HA titer when drug was used at a concentration of 50mg/kg. Similarly **BinHafeez et al (2003)** also showed increased HA tire at doses of 50 mg/kg and above, of fenugreek extract administered on mice. The study showed that up to 300mg/kg of the crude

drug could enhance the humoral immune response. Upon examining the present results it is evident that the ethanolic extract at concentrations less than 500 mg/kg induces humoral immune response as evidenced by HA titer.

One of the earliest immune response can be seen and measured by studying the hematological parameters of an animal. Accordingly parameters like total leukocyte count for control group as well as group which received various concentrations of drug. Blood cells are the first cells to be responding to invading non self materials. An immunomodulatory effect of any immune substance would first see as a change in leukocyte count. In the present study group IV which received (50 mg/kg) of standard drug showed highest leukocyte count of  $15.01 \times 10^3$  cu.mm showing the initial triggering of blood cell to mount a potent immune response. The results showing standard drug concentration are better to elicit good immune response than test concentrations (100 mg/kg, 500mg/kg) of drug administered. The results are further strengthened with highest percentage of neutrophil being circulated in the group.

Serum protein is one of the earliest indicators of normal serum chemistry of an individual. A change in serum protein concentration and albumin ratio would hint us about the altered immune response status of the individual. Accordingly in the present study serum protein level is found to be similar in case of control and higher concentration (500 mg/kg) of the drug but in the lower concentration 100 mg/kg of drug test the group showed increase in serum protein showing that higher immune response might have contributed to the serum protein in terms of different molecules such as immunoglobulin and other humoral factors. The serum protein increased in case of Levamisole 50 mg/kg administered by oral.

## 9. CONCLUSION

- ✚ Many of the *Annona* species have been reported for immunomodulatory activity. However, *Annona reticulata* fruits not been reported for the same.
- ✚ Phytochemical studies showed alkaloids, Phenolics, Flavanoids, carbohydrate, terpenoids, steroids, and saponins.
- ✚ Acute toxicity study was done and no mortality reported at doses between 50 mg/kg to 500 mg/kg.
- ✚ Determined Delayed type hypersensitivity response show significance paw volume of lower and higher concentration as compared to control.
- ✚ Evaluation of Humoral antibody titre value showed highly significant increase in antibody titre values compared to control group
- ✚ Determined the low dose of extract (100 mg/kg) show effect on TLC count compared to control group
- ✚ Estimation of total serum protein show significant increase of total serum value compared to control.
- ✚ Ethanolic extract of fruits of *Annona reticulata* showed moderate immunomodulatory activity compared with standard drug.

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